REMARKS

Claims 16, 17, and 21-28 are pending in the instant application. Claims 16, 17, and 21-28 have been rejected. No new matter has been added by this amendment. Reconsideration is respectfully requested in light of the following remarks.

I. Rejection Under 35 U.S.C. §112

Claims 16, 17, and 21-28 are rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement. Specifically, the Examiner suggests that Applicants have not provided an adequate description of the genus of antibodies having the capability of recognizing a naturally conformed extracellular portion of BCRP. Due to the means by which Applicants have produced the claimed antibody, the Examiner suggests that the claimed antibody does not appear to be conventional in the art, and that Applicants should provide an adequate written description to teach the structures of the antibody that allows recognition of the extracellular portion of the BCRP in its natural conformation. Applicants respectfully traverse this rejection.

Compliance with written description requirement of 35 U.S.C. 112, first paragraph, may be shown by any description of sufficient, relevant, identifying characteristics so long as a person skilled in the art would recognize that the inventor had possession of the claimed invention. For some biomolecules, examples of identifying characteristics include a sequence, structure, binding affinity, *binding specificity*, molecular weight, and length. Although structural formulas provide a convenient method of demonstrating possession of specific

molecules, other identifying characteristics or combinations of characteristics may demonstrate the requisite possession. For example, disclosure of an antigen fully characterized by its structure, formula, chemical name, physical properties, or deposit in a public depository provides an adequate written description of an antibody claimed by its binding affinity to that antigen. *Noelle v. Lederman*, 355 F.3d 1343, 1349, 69 USPQ2d 1508, 1514 (Fed. Cir. 2004) (holding there is a lack of written descriptive support for an antibody defined by its binding affinity to an antigen that itself was not adequately described). See MPEP §2163.

The amino acid sequence and characterization of BCRP as an ABC transporter was well-established in the art at the time of filing of the instant application. Applicants clearly provide at page 39 of the instant application the detailed steps which can be, and were, carried out to overexpress a known BCRP nucleotide sequence in a living cell to produce an antibody to a naturally conformed extracellular portion of BCRP, *i.e.*, a BCRP having the native three-dimensional structure such that it forms a homodimer with the appropriate extracellular domain exposed on the cell surface.

As would be readily apparent to the skilled artisan upon reading the instant disclosure, Applicants appreciated the necessity of producing BCRP in its natural conformation in living cells to increase the probability that the immune system would detect external BCRP epitopes in their native configuration, rather than epitopes that were internally located in the cells, or epitopes only present in denatured protein (see page 23, lines 11-16). While the antigen used to induce an immune response was,

in fact, a cell with BCRP epitopes exposed on the cell surface, the antibodies produced had the same well-characterized structure of any other conventional antibody (*i.e.,* an effector portion which is the constant region and a variable region that contains the antigen binding sites in the form of complementarity determining regions and the framework regions) because the antibodies were produced by the mammalian immune system (see page 39, line 12) having constant and framework region sequences known and published in the art. Therefore, the distinguishing identifying characteristic sufficient to show that Applicants were in possession of the claimed antibody is the antigen specificity of the claimed antibody, *i.e.,* an antibody raised against an extracellular portion of a naturally conformed BCRP (see page 39, lines 12-23). Using this art-accepted characterization of an antibody, Applicants believe they have met the requirement under 35 USC 112, first paragraph, and it is therefore respectfully requested that this rejection be reconsidered and withdrawn.

Claims 16, 17, and 21-28 are also rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. It is suggested that the method taught in page 39 of the specification is well-known in the art as taught by *Mechetner et al.* (U.S. Patent No. 5,994,088), but Applicants have argued that the art known methods at the time of the invention could not predictably produce the claimed antibody. The Examiner further suggests that the specification fails to disclose whether the antibody produced

by the disclosed method had indeed been used in the detection of the BCRP expression in living cells. Further, the Examiner suggests that detection of BCRP expression in living cells is not sufficient evidence that an antibody made by Applicants recognizes an external epitope on BCRP in its natural conformation because an antibody made with a purified protein or a synthetic antibody could also be used to detect an extracellular epitope in its natural form as long as it recognizes a part of the epitope. It is suggested that because antibody-antigen recognition is largely conformational independent, as taught by *Niman et al.* (U.S. Patent No. 5,563,247), that an antibody would detect an external epitope in its natural conformation as long as it can recognize a part of the entire conformation. Applicants respectfully traverse this rejection.

At the outset, Applicants respectfully disagree with the Examiners conclusions, based upon the teachings of *Niman et al.*, that antibody-antigen recognition is largely conformational independent and that an antibody would detect an external epitope in its natural conformation as long as it can recognize a part of the entire conformation. At the time of filing it was known in the art that conformational epitopes existed and that not all antibodies produced against a linear epitope would recognize a conformational epitope and, likewise, not all antibodies produced against a conformation epitope would recognize a linear epitope. For example, *Munodzana, et al.* ((1998) *Infect. Immun.* 66:2619-2624; enclosed herewith) teach that antibodies to multimeric outer membrane MSPs of *A. marginale* will only recognize MSPs when the native secondary and tertiary structure has been adopted (see

abstract). Further, this reference teaches that isolated, recombinant-expressed MSPs, either alone or in combination, fail to induce antibody-mediated protective immunity compared to the antibody-mediated protective immunity induced with whole outer membranes or a native protein complex (see page 2619, first column). Therefore, the antibody-antigen recognition process can be conformational dependent, wherein secondary, tertiary and quaternary structures may be necessary to generate an antibody which recognizes a particular domain and conformation.

MPEP 2164.04 states that a specification disclosure which contains a teaching of the manner and process of making and using an invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as being in compliance with the enablement requirement of 35 U.S.C. 112, first paragraph, unless there is a reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.

As previously indicated at page 10 of Applicants' reply filed July 29, 2004, page 39 of the instant application clearly describes each step used *to make* an antibody which recognizes an extracellular portion of a naturally conformed BCRP and page 24, lines 19-25 teaches how *to use* an antibody which recognizes an extracellular portion of a naturally conformed BCRP. The identification of an antibody that recognizes an extracellular portion of a naturally conformed BCRP was carried out by Applicants by taking supernatants from each hybridoma clone and screening them by flow cytometry using a living human breast cancer cell line (MCF-7) that had been transduced with an amphotrophic HaBCRP vector. Any supernatant that showed

reactivity in this assay was then back-screened on the parental MCF-7 line, and clones that reacted with the living MCF-7 HaBCRP cells but not with the parental MCF-7 line were scored as positive and specific. These cells were subcloned and re-screened based on the indicator cell lines. See page 39, lines 17-23. Thus, one of skill would reasonably conclude that an antibody which binds to the surface of a living cell transduced with an amphotrophic HaBCRP vector and not to a living cell lacking the amphotrophic HaBCRP vector is an antibody which recognizes an extracellular portion of BCRP in its natural conformation as BCRP is a plasma membrane-bound protein with the extracellular portion exposed on the cell surface and antibodies are large proteins which would not cross the plasma membrane and bind to any other portions of BCRP (*e.g.*, the transmembrane or intracellular domains).

Further, Applicants submit herewith a corroborative Declaration by Dr. Sorrentino which indicates that, in addition to the 5D3 antibody disclosed by *Zhou et al.* and *Abbott et al.* (referred to in Applicants' reply filed July 29, 2004), Applicants have generated three other independent monoclonal antibodies that recognize an extracellular portion of BCRP in its natural conformation. Like 5D3, these antibodies, designated 7A3, 1C5 and 8C2, were generated using the same methodology as disclosed in the instant application and each recognizes an extracellular portion of BCRP in its natural conformation in living cells (*i.e.*, human myeloid leukemic cell line AML3 over-expressing BCRP).

Accordingly, the method disclosed in the instant application is a *validated, reliable* method for producing any antibody which

recognizes an extracellular portion of BCRP in its natural conformation as the BCRP has adopted its proper secondary, tertiary and quaternary structures when produced in living cells. As the instant specification has described a manner and process of making and using the claimed antibodies, the specification is in compliance with the enablement requirement of 35 U.S.C. 112, first paragraph. It is therefore respectfully requested that this rejection be reconsidered and withdrawn.

II. Rejection Under 35 U.S.C. §103

Claims 16, 17, and 21-28 have been rejected under 35 U.S.C. 103(a) as being unpatentable over *Ross et al.* (U.S. Patent No. 6,313,277) in view of *Mechetner et al.* (U.S. Patent No. 5,994,088).

The Examiner suggests that the '088 patent discloses the same method as the instant specification for generating antibodies that will recognize the extracellular portion of an ABC transporter. It is suggested that with the knowledge of the BCRP coding sequence as taught by *Ross et al.* and the known method for producing antibodies recognizing an external epitope of an ABC transporter protein as taught by *Mechetner et al.*, one of skill in the art would have a reasonable expectation of success for producing an antibody as suggested by *Ross et al.* that could recognize an external epitope of BCRP in its natural conformation. The Examiner further suggests that given the knowledge regarding the importance of the extracellular portion of an ABC transporter protein, and given the cDNA of BCRP provided by *Ross et al.*, it is within the knowledge of the skilled artisan to make a similar antibody as 4E3 or UIC2 that

binds to the naturally conformed extracellular epitope of BCRP as taught by *Mechetner et al.*

Applicants respectfully traverse this rejection. The cited references fail to meet all the basic criteria set forth to establish a *prima facie* case of obviousness. MPEP §2143. As previously indicated and acknowledged by the Examiner, Ross does not teach or suggest an antibody that recognizes the extracellular portion of BCRP in its natural conformation. Likewise, *Mechetner et al.* do not teach or suggest an antibody that recognizes the extracellular portion of BCRP in its natural conformation.

Applicants maintain that the teachings of *Mechetner et al.* do not overcome the general uncertainty in the prior art when it comes to the ability to generate antibodies that will recognize the extracellular portion of an ABC transporter in its native conformation as this superfamily of proteins is very large and diverse; some ABC transporters are single protein molecules, while others are homo- or heterodimers. As indicated in the Supplemental Declaration by Dr. Sarkadi, submitted herewith, the use of any particular method for producing an antibody to an ABC transporter must be determined on a case-by-case basis. Only when an antibody is actually produced to a particular ABC transporter can the method used be validated for the production of an antibody to that particular ABC transporter, in particular when a specific domain (*e.g.*, extracellular portion) and conformation (*e.g.*, the natural conformation) is desired.

Unlike the P-glycoprotein disclosed by *Mechetner et al.*, which is a single polypeptide of two homologous halves, BCRP is a homodimer wherein two half-transporter molecules must come

together to establish a fully functional protein. Therefore, the quaternary protein structure (*i.e.*, clustering of several individual peptides into a final, specific conformation) of BCRP is a distinct feature not appreciated in the teachings of the method of *Mechetner et al.* and therefore one of skill in the art could not reasonably predict from the teachings of *Mechetner et al.* that the method used to produce an antibody that recognizes an extracellular epitope of Pgp in its biochemical conformation as a single protein molecule will successfully produce an antibody to an extracellular portion of BCRP in its natural conformation where it is a half-transporter that forms a homodimer.

It has been consistently held that the fact that a certain result or characteristic may occur or be present in the prior art is not sufficient to establish the inherency of that result or characteristic. *In re Rijckaert*, 9 F.3d 1531, 1534, 28 USPQ2d 1955, 1957 (Fed. Cir. 1993)(reversed rejection because inherency was based on what would result due to optimization of conditions, not what was necessarily present in the prior art); *In re Oelrich*, 666 F.2d 578, 581-82, 212 USPQ 323, 326 (CCPA 1981). Neither alone nor combined do Ross or *Mechetner et al.* teach or suggest the effective generation of an isolated antibody which recognizes an extracellular portion of BCRP in its natural conformation. Further, neither cited reference provides to one of ordinary skill in the art a reasonable expectation of success in achieving the instant invention. Therefore, the cited references fail to make the instant invention obvious and it is therefore respectfully requested that this rejection be withdrawn.

III. Conclusion

The Applicants believe that the foregoing comprises a full and complete response to the Office Action of record. Accordingly, favorable reconsideration and subsequent allowance of the pending claims is earnestly solicited.

Respectfully submitted,

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Conformational Dependence of *Anaplasma marginale* Major Surface Protein 5 Surface-Exposed B-Cell Epitopes†

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The *Anaplasma marginale* outer membrane is composed of immunogenic major surface proteins (MSPs) linked both covalently and noncovalently in multimeric complexes (M. C. Vidotto, T. C. McGuire, T. F. McElwain, G. H. Palmer, and D. P. Knowles, Infect. Immun. 62:2940–2946). Consequently, effective induction of antibody against surface-exposed MSP epitopes has been postulated to require maintenance of MSP secondary through quatenary structures. Using MSP5 as a model and the approach of epitope mapping with recombinant expressed full-length and truncated proteins, we demonstrated that the immunodominant surface epitope bound by monoclonal antibody (MAb) ANAF16C1 required disparate amino- and carboxy-terminal regions of MSP5, indicating the conformational dependence of this epitope. The required amino-terminal MSP5 region included the cysteines involved in intramolecular disulfide bonding. The dependence of the immunodominant epitope on disulfide bonding was confirmed by loss of MAb ANAF16C1 binding to MSP5 following disulfide bond reduction and covalent modification of the reduced sulfhydryl groups. The recognition of the MSP5 immunodominant epitope by antibody induced by protective immunization with *A. marginale* outer membranes was also conformationally dependent, as shown by the loss of epitope binding following serum adsorption with native but not reduced and denatured *A. marginale*. Importantly, the antibody response to all immunodominant MSP5 surface epitopes was restricted to conformationally dependent epitopes, since the binding of polyclonal anti-MSP5 antibody to the *A. marginale* surface could be blocked by adsorption with native but not denatured and reduced MSP5. These results confirm the importance of the secondary and tertiary structures of MSP epitopes as immune system targets and support the testing of immunogens which maintain the required conformation.

Anaplasma marginale is an arthropod-borne ehrlichial pathogen of cattle that invades and replicates in mature erythrocytes (7). Acute infection is characterized by high levels of rickettsemia (>10^9 infected erythrocytes/ml) and severe anemia, which frequently results in abortion or death (5, 7). Immunity against acute *A. marginale* rickettsemia is directed against outer membrane surface proteins, and infectivity can be neutralized with antibodies against surface exposed epitopes (18, 20, 21). Correspondingly, cattle immunized with *A. marginale* outer membranes develop significantly lower rickettsemia following challenge than do adjuvant-immunized controls (20, 22, 27). Sera from these immunized and protected cattle recognize six major surface proteins (MSPs), and antibody titers against MSP2 and MSP5 correlate with protection against challenge with the homologous strain (20, 22, 27). In contrast to protection induced by immunization with whole outer membranes or a native MSP1a/MSP1b complex, isolated recombinant-expressed MSPs, either alone or in combination, fail to induce comparable protection against rickettsemia (17, 18, 20, 27). Consequently, we have hypothesized that MSP conformation, as determined by secondary through quatenary structures, is a critical determinant in the efficacy of experimental vaccines (13, 20, 30).

The outer membrane is composed of MSPs linked both covalently and noncovalently in multimeric complexes (30). MSP5 and MSP2 occur in both monomeric intramolecularly disulfide-bonded and multimeric intermolecularly disulfide-bonded forms in the membrane: MSP5 as a dimer and MSP2 as a tetramer (19, 30, 31). Importantly, both MSP5 and MSP2 bear immunodominant B-cell epitopes and, in outer membrane-immunized cattle, the antibody titer correlates with protection against challenge with the homologous *A. marginale* strain (19, 27, 31). Based on our hypothesis, we would predict that the MSP2 and MSP5 immunodominant surface-exposed epitopes are conformationally dependent and require disulfide bonding to maintain epitope conformation. We chose to first test this prediction with intramolecularly disulfide bonded MSP5. MSP5, in contrast to the antigenically variable MSP2 (2, 19), is encoded by a single highly conserved gene and expresses invariant surface epitopes recognized by outer membrane-immunized as well as previously infected immune cattle (1, 6, 14). In this paper, we report the disulfide bond and conformational requirements of defined MSP5 surface-exposed epitopes and the results of testing whether antibody binding to the *A. marginale* surface requires maintenance of secondary and tertiary structures.

MATERIALS AND METHODS

Physical mapping. ANAF16C1 is an immunoglobulin G1 (IgG1) monoclonal antibody (MAb) directed against the *A. marginale* surface and binds MSP5 in all strains of *A. marginale*, *A. ovis*, and *A. centrale* tested (1, 6, 12, 14). *Escherichia coli* transformed with plasmid pAM104A expresses a full-length MSP5 polypeptide that is bound by MAb ANAF16C1 (31). Full-length and truncated *msp5* clones expressed as fusion partners with maltose binding protein (MBP) were used to identify the MSP5 region bound by MAb ANAF16C1. Briefly, the entire

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† This paper is dedicated to the memory of Devere Munodzana and to his family.

‡ Deceased.

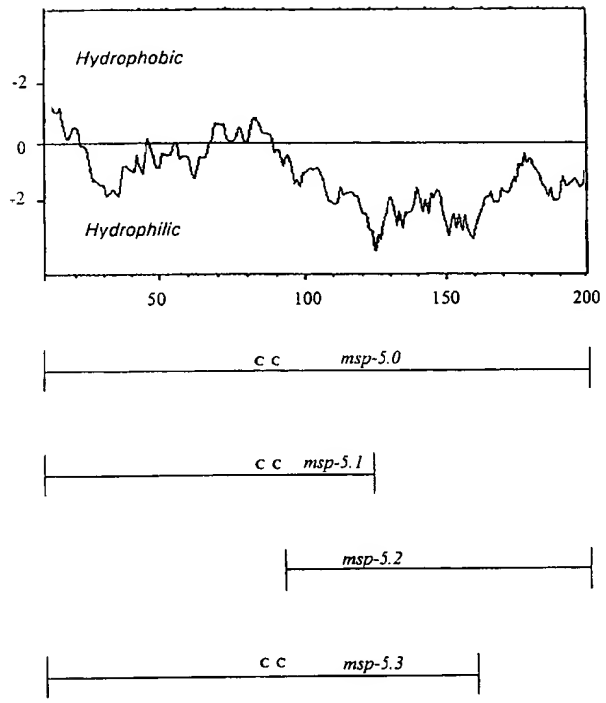

FIG. 1. Physical map of the recombinant MSP5 proteins relative to a transmembrane helical protein hydrophobicity-hydrophilicity profile of native MSP5. The map was generated with the Genetics Computer Group package from the University of Wisconsin. The *y* axis reflects the Goldman-Engelman-Steitz hydrophobicity scale over a window of 20 residues, and the *x* axis represents the amino acid position in MSP5. The proteins expressed by full-length (*msp-5.0*) or truncated (*msp-5.1*, *msp-5.2*, and *msp-5.3*) recombinant clones are plotted against the same *x* axis, and the positions of the two cysteines are indicated by the letter C.

msp5 open reading frame (nucleotides 118 to 753 based on the numbering of the original clone in pAM104A [31]) was amplified with forward and reverse primers incorporating *Xba*I recognition sites, digested, and ligated in frame into the *Xba*I site of the vector pMal-c2 (24). The plasmid encoding the full-length MSP5-MBP fusion was designated *msp5.0*, and the expressed protein was designated MSP5.0. The following truncated *msp5* clones were generated by the same strategy with site-specific forward and reverse primers: *msp5.1*, a 371-bp clone representing bp 118 to 488; *msp5.2*, a 356-bp clone representing bp 390 to 745; and *msp5.3*, a 483-bp clone representing bp 118 to 600. The sequences of all clones were verified by double-strand sequencing by primer extension with dideoxy chain termination (25). *E. coli* XL-1 Blue was transformed with each plasmid, and the expression of an MSP5-MBP fusion protein of the appropriate size was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of recombinant *E. coli* lysate and immunoblotting with detection by rabbit anti-MBP polyclonal antibody (3). The orientations of the full-length and truncated *msp5* constructs and the encoded proteins relative to the predicted conformation of native MSP5 are shown in Fig. 1. Each MSP5-MBP fusion protein was purified on individual amylose affinity columns following extraction as soluble proteins from recombinant *E. coli* (24). Briefly, 2×10^8 bacteria per ml of rich medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.2% glucose), containing 100 μg of ampicillin per ml, was incubated in the same medium with the addition of 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h at 37°C to induce fusion protein expression. Bacteria were disrupted by freezing and rapid thawing followed by sonication. The recombinant expressed proteins were collected in the supernatant and loaded on amylose columns with a binding capacity of 3 mg of MBP per ml of resin. The columns were washed and the recombinant fusion proteins were eluted as previously described (24). Eluted recombinant proteins were detected by immunoblotting with rabbit anti-MBP polyclonal antibody and then tested for reactivity with MAb ANAF16C1 by SDS-PAGE and immunoblotting (3). Antibody binding was detected by using horseradish peroxidase-labeled goat anti-rabbit IgG (for anti-MBP antibody) or goat anti-murine IgG (for MAbs) and enhanced chemiluminescence (3). Purified nonfusion MBP, and unrelated MBP fusion protein (MBP–*Babesia bovis* RAP-1 [26]), and uninfected erythrocytes were used as negative control antigens. *A. marginale*-infected erythrocytes and *E. coli* transformed with plasmid pAM104A were used as positive antigen controls (31). Normal rabbit serum and the IgG1 MAb Trypl E1 were used as negative antibody controls.

Conformational sensitivity of MAb ANAF16C1 binding. Affinity-purified MSP5.0 was incubated, at 10 μg per treatment (in duplicate), with either 8 M urea, 60 mM dithiothreitol (DTT), or 300 mM iodoacetamide (IA), or one of the combinations DTT and IA; urea and IA; or urea, DTT, and IA. The urea and DTT treatments were performed at 56°C for 12 h, and the IA treatment was performed for 1 h at 25°C (4). An untreated sample was incubated identically and used as a positive control. Reactivity was determined by immunoblotting (3) with MAb ANAF16C1 or the negative control MAb Trypl E1.

Cattle previously immunized with purified *A. marginale* outer membranes developed high titers of anti-MSP5 antibody and were shown to be protected against acute rickettsemia upon challenge (27, 31). Serum obtained postimmunization but prechallenge was adsorbed with either denatured and reduced (8 M urea, 60 mM DTT, 300 mM IA) or untreated, native Norton strain organisms (28). As controls, serum either was left unadsorbed or was adsorbed by the identical method with either denatured and reduced *E. coli* or untreated *E. coli*. Adsorption, performed at 25°C for 1 h, was repeated until there was no reactivity with the adsorbing antigen preparation as determined by immunoblotting. Each serum treatment was then tested for inhibition of MAb ANAF16C1 binding to recombinant MSP5.0 by a competitive inhibition enzyme-linked immunosorbent assay (ELISA) as described previously (1, 6). Briefly, individual wells in 96-well plates were coated with 1 μg of amylose-resin-purified MSP5.0 fusion protein in 100 μl of carbonate-bicarbonate coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$ [pH 9.6]). The wells were incubated for 1 h at room temperature with 200 μl of blocking buffer (250 mM K_2HPO_4, 250 mM KH_2PO_4, 0.5% fraction V bovine serum albumin, 0.75% glycine, 1% sucrose) and then washed four times with phosphate-buffered saline (PBS; pH 7.2). The adsorbed and unadsorbed test sera were diluted in PBS–1% BSA, to a final dilution of 1:40,000, the dilution of unadsorbed serum that resulted in approximately 70% inhibition of MAb ANAF16C1 binding to MSP5.0. Adsorbed, diluted sera were added to triplicate wells in 100-μl aliquots, and the wells were incubated at room temperature for 1 h. The wells were washed four times with 200 μl of PBS per well and then incubated for 15 min at room temperature with horseradish peroxidase-conjugated MAb ANAF16C1 as described previously (1, 6). After four additional washes with PBS, 50 μl of 0.5-μg/μl *o*-phenylenediamine hydrochloride dihydrochloride in substrate buffer (0.2 M Na_2HPO_4, 0.1 M citric acid) was added to each well. The plates were incubated for 10 min, and the reactions were terminated with 25 μl of 2 N H_2SO_4. The results were expressed as percent inhibition (and standard deviation) of MAb ANAF16C1 binding to MSP5.0 (1, 6).

Conformational dependence of antibody binding to *A. marginale* MSP5 surface exposed epitopes. Calves were obtained at 1 day of age and raised in a tick- and fly-free facility at the Central Veterinary Laboratory, Harare, Zimbabwe. Before immunization, sera were shown to be unreactive with *A. marginale* by immunoblotting against whole-organism lysate (11) and by the competitive inhibition MSP-5.0 ELISA (1, 6). Five calves were immunized by subcutaneous inoculation of 50 μg of native MSP5, purified from *A. marginale* on a MAb ANAF16C1 affinity column as described previously (31), in saponin adjuvant. The immunization was repeated three times at 3- to 4-week intervals. Five adjuvant control calves were given saponin alone by using the identical schedule and route of inoculation. Sera were obtained 1 month after the last inoculation, and the anti-MSP5 titer was determined by the competitive inhibition ELISA. As described in Results, all sera from MSP5-immunized calves had high titers of anti-MSP5 antibody. Two of these sera were then adsorbed with amylose resin-purified MSP5.0 or denatured and reduced (8 M urea, 60 mM DTT, 300 mM IA) purified MSP5.0. As controls, these sera either were left unadsorbed or were adsorbed, by using the identical protocol, with either denatured and reduced MBP or untreated MBP. Adsorptions, performed at 25°C for 1 h, were repeated until there was no reactivity with the adsorbing antigen preparation as determined by immunoblotting. Unadsorbed and adsorbed sera were then tested for binding to native surface exposed MSP5 epitopes by agglutination of purified *A. marginale* as previously described (19).

RESULTS

Physical mapping. The physical maps of the full-length and truncated MSP5-MBP fusion proteins expressed in pMal-c2 are shown in Fig. 1. Each fusion protein was purified on individual amylose affinity columns and identified by SDS-PAGE and immunoblotting with detection by rabbit polyclonal antibody specific for the MBP fusion partner. Figure 2 shows the binding of anti-MBP antibody to the expected 65-kDa MSP5.0 fusion protein (lane 1) and to MBP alone (lane 2). The anti-MBP antibody also reacted with the truncated fusion proteins MSP5.1, MSP5.2, and MSP5.3 (data not shown) but not with purified *A. marginale* (lane 3). There was no binding of control normal rabbit sera to any of the MSP5-MBP fusion proteins

FIG. 2. Expression of recombinant MSP5.0. Lanes 1 and 4 contain MSP5.0 fused to MBP and purified on an amylose affinity column; lanes 2 and 5 contain MBP alone; and lanes 3 and 6 contain a lysate of *A. marginale*-infected erythrocytes. Lanes 1 to 3 were reacted with rabbit antiserum against MBP; lanes 4 to 6 were reacted with the same dilution of normal rabbit serum. The positions of the MSP5.0-MBP fusion protein and MBP alone are indicated in the left margin.

(MSP5.0 is shown in lane 4), MBP (lane 5), or *A. marginale* (lane 6).

Each recombinant MSP5 fusion protein was then tested for reactivity with MAb ANAF16C1 or the Trypl E1-negative control MAb by immunoblotting. MSP5.0 was bound by MAb ANAF16C1 (Fig. 3, lanes 2 and 3) but not by an isotype control MAb, Trypl E1 (lanes 6 and 7). This indicates that the presence of the MBP fusion partner does not alter recognition of the MSP5 epitope by MAb ANAF16C1. This MAb also bound *A. marginale* native MSP5 (lane 4). MAb ANAF16C1 did not react with the negative control *B. bovis* RAP-1–MBP fusion protein (lane 1). Of the truncated fusion proteins, only MSP5.3 was bound by MAb ANAF16C1 (Fig. 4, lane 4). ANAF16C1 did not bind MSP5.1 (lane 2), MSP5.2 (lane 3), or the negative control *B. bovis* RAP-1–MBP fusion protein (lane 1). This reactivity indicates that not only is the amino-terminal region (nucleotides 118 to 390, encoding the first 91 amino acids including the conserved cysteine residues) necessary for ANAF16C1 binding but that also some or all of the region composed of amino acids 125 to 161 (encoded by nucleotides 492 to 600) is also required. These data, without further mapping, are consistent with conformational dependence of the immunodominant epitope bound by MAb ANAF16C1. Nonfusion MSP5 expressed by *E. coli* containing plasmid p104A was used as a positive

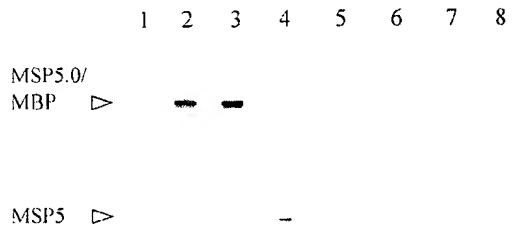

FIG. 3. MAb ANAF16C1 binds recombinant MSP5.0. Lanes 1 and 5 contain *B. bovis* RAP-1–MBP fusion protein as a negative antigen control; lanes 2, 3, 6, and 7 contain MSP5.0-MBP fusion protein (lanes 2 and 6 contain protein from a different column fraction from the protein in lanes 3 and 7); lanes 4 and 8 contain a lysate of *A. marginale*-infected erythrocytes. Lanes 1 to 4 were reacted with MAb ANAF16C1; lanes 5 to 8 were reacted with the isotype control MAb Trypl E1. The positions of the MSP5.0-MBP fusion protein and the native MSP5 are indicated in the left margin.

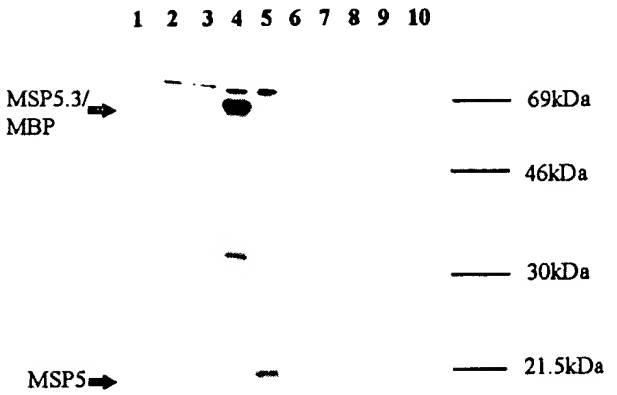

FIG. 4. MAb ANAF16C1 binds recombinant MSP5.3 but not MSP5.1 or MSP5.2. Lanes 1 and 6 contain *B. bovis* RAP-1–MBP fusion protein as a negative antigen control; lanes 2 and 7 contain MSP5.1-MBP fusion protein; lanes 3 and 8 contain MSP5.2-MBP fusion protein; lanes 4 and 9 contain MSP5.3-MBP fusion protein; and lanes 5 and 10 contain nonfusion MSP5 expressed by *E. coli* containing plasmid p104A (31). Lanes 1 to 5 were reacted with MAb ANAF16C1; lanes 6 to 10 were reacted with the isotype control MAb Trypl E1. The positions of the MSP5.3-MBP fusion protein and nonfusion MSP5 are indicated in the left margin.

control and was bound, as expected, by MAb ANAF16C1 (Fig. 4, lane 5).

Conformational sensitivity of MAb ANAF16C1 binding. The conformational dependence of MSP5 was tested by treatment of purified MSP5.0 with denaturing and reducing agents followed by determination of MAb ANAF16C1 binding. Reduction of disulfide bonds with DTT followed by covalent modification of sulfhydryl groups with IA to prevent reoxidation completely abolished MAb binding (Fig. 5). This effect was probably due to the effect on disulfide bonding, since neither DTT nor IA alone had any detectable effect on the epitope (Fig. 5). This dependence on disulfide bonding is consistent with the epitope-mapping results, which showed a requirement for the amino-terminal half of MSP5, containing the conserved

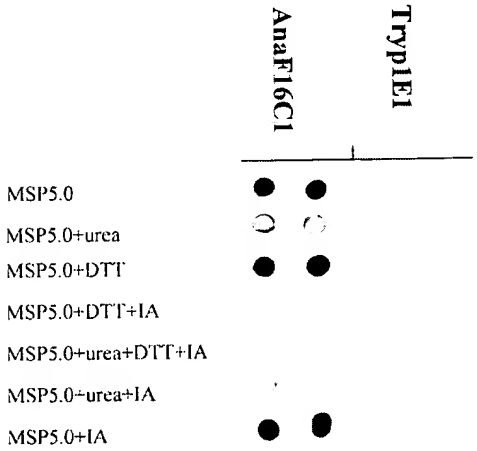

FIG. 5. MAb ANAF16C1 binding is sensitive to denaturation and reduction of MSP5. Purified MSP5.0 was either untreated or treated with 8 M urea (MSP5.0+urea); 60 mM dithiothreitol (MSP5.0+DTT); DTT and 300 mM IA (MSP5.0+DTT+IA); urea, DTT, and IA (MSP5.0+urea+DTT+IA); urea and IA (MSP5.0+urea+IA); or IA alone (MSP5.0+IA). Samples were reacted in duplicate with either MAb ANAF16C1 (left) or the negative control MAb Trypl E1 (right).

TABLE 1. Binding of antibody induced by outer membrane immunization to the MSP5 immunodominant epitope is dependent on native conformation of *A. marginale*

Treatment of anti-outer membrane serum	% Inhibition of MAb ANAF16C1 binding[a]
Unadsorbed	70 ± 5
Adsorbed with denatured *E. coli* lysate	60 ± 11
Adsorbed with native *E. coli* lysate	63 ± 8
Adsorbed with denatured *A. marginale* lysate	56 ± 13
Adsorbed with native *A. marginale* lysate	20 ± 10

[a] Negative control serum from a nonimmunized, uninfected calf gave a background inhibition of 12 ± 3%.

cysteine residues. Treatment with 8 M urea, which denatures the protein secondary structure, resulted in a partial loss of MAb ANAF16C1 binding (Fig. 5). This effect is again consistent with a conformationally dependent epitope and may involve both the amino- and carboxy-terminal hydrophilic regions.

To test whether recognition of the MSP5 immunodominant epitope by antibody from outer membrane-immunized and protected cattle was also conformationally dependent, serum was adsorbed with native or reduced and denatured *A. marginale* lysate and then tested for the ability to inhibit MAb ANAF16C1 binding. Unadsorbed serum was diluted (1:40,000) to achieve 70% inhibition of MAb ANAF16C1 binding (Table 1). All test samples following adsorption were then tested at a final dilution of 1:40,000. As shown in Table 1, adsorption with native *A. marginale* significantly depleted bovine serum antibody inhibition of MAb ANAF16C1 binding. In contrast, adsorption with native *E. coli* or reduced and denatured *A. marginale* or *E. coli* did not significantly reduce the binding of the immune bovine serum to the MSP5 immunodominant epitope (Table 1). This indicates that the antibody response to this MSP5 epitope following effective outer membrane immunization is conformationally restricted.

Conformational dependence of antibody binding to *A. marginale* MSP5 surface-exposed epitopes. Immunization of cattle with native MSP5 induced high titers of antibody against the immunodominant MSP5 epitope, as determined by the competitive inhibition ELISA based on ANAF16C1 binding (data not shown). Sera from two of the MSP5-immunized cattle were then used to determine if recognition of MSP5 epitopes on the *A. marginale* surface was conformationally dependent. Unadsorbed sera had end-point agglutination titers of 512, while adsorption with native MSP5.0 diminished surface binding 32- and 64-fold, respectively, for each of the two test sera (Table 2). In contrast, adsorption with denatured and reduced MSP5.0 lysate either did not alter (anti-MSP5.0 serum 1) or only slightly diminished (anti-MSP5.0 serum 2) surface reactivity compared to negative control adsorptions with either native or reduced and denatured MBP (Table 2). Sera from the five cattle immunized with saponin alone had end-point agglutination titers of 4 or less (data not shown). These results indicate that the antibody response to MSP5, as presented on the *A. marginale* surface, is predominantly against conformationally dependent epitopes.

DISCUSSION

Why individual MSPs fail to induce protection at a level comparable to that induced by immunization with intact *A. marginale* outer membranes is unknown and represents an important gap in our knowledge needed to develop and improve vaccines against ehrlichial pathogens. Possible explanations, which are not mutually exclusive, include the following: (i) each MSP alone induces partially protective immunity, and the efficacy of the outer membrane complex simply reflects the sum of the individual components; (ii) the multimeric outer membrane complex enhances antigen presentation compared to soluble individual MSPs and generates a phenotypically different immune response; and (iii) induction of protection requires antibody to conformationally dependent epitopes on the *A. marginale* surface. The first possibility is not congruent with data showing that combinations of up to three MSPs do not consistently enhance protection compared to that afforded by immunization with individual MSPs (17, 18, 20, 23). In contrast, both the second and third explanations remain viable. As an entrée to investigating the importance of antibody against conformationally dependent epitopes, we analyzed the structural requirements of a highly conserved immunodominant epitope on MSP5. This epitope, defined by binding of MAb ANAF16C1, is conserved among all tested strains of *A. marginale*, *A. centrale*, and *A. ovis* and induces high titers of antibody in all infected species including cattle, sheep, and goats (1, 6, 14, 31). Initial physical mapping of the epitope with full-length and truncated recombinant expressed MSP5 indicated that residues encoded 5′ to nucleotide 390 (amino acid 91) as well as some or all of the region encoded by nucleotides 492 to 600 (amino acids 125 to 161) were required. Importantly, the required amino-terminal region included the conserved cysteines (31), consistent with the proposed importance of intramolecular disulfide bonding in the MSP conformation (30). The absolute dependence of the immunodominant epitope on disulfide bonding was confirmed by the loss of MAb ANAF16C1 binding to MSP5.0 following disulfide bond reduction and covalent modification of the reduced sulfhydryl groups. Interestingly, MAb binding was also reduced after urea treatment alone (Fig. 5). This suggests that secondary protein structure, apart from the tertiary requirements for intramolecular disulfide bonding, is also needed for epitope conformation, a finding consistent with the physical mapping results indicating contributions from two distant hydrophilic regions of MSP5. Whether amino acids in these disparate regions are juxtaposed to form the actual epitope (defined by binding to the complementarity determining regions of the antibody) or whether the epitope is encoded within one of the regions and the second is required only to provide correct secondary structure for binding is unknown. Both scenarios are consistent with the requirement for disulfide bonding in or adjacent to a hydrophobic segment interposed between two hydrophilic and presumed surface-exposed regions of MSP5 (Fig. 1).

The single MSP5 epitope defined by MAb ANAF16C1 binding was analyzed as a model for immunodominant MSP epitopes (11, 20, 30). The presence of conserved cysteines

TABLE 2. Binding of anti-MSP5 sera to the *A. marginale* surface requires reactivity with native, nondenatured epitopes

Treatment of anti-MSP5 serum	End-point agglutination titer for:	
	Serum 1	Serum 2
Unadsorbed	512	512
Adsorbed with denatured MBP	256	512
Adsorbed with native MBP	256	256
Adsorbed with denatured MSP5.0	256	128
Adsorbed with native MSP5.0	16	8

and disulfide bonds in MSP2 and MSP4 (15, 19, 30) suggests that conformational dependence may be a common feature among *A. marginale* MSPs. In addition, the presence of an MSP5 homolog in *Cowdria ruminantium* MAP-2 (8) and of MSP2 homologs in *C. ruminantium* MAP1 (19, 29) and *Ehrlichia chaffeenesis* OMP-1 (16) provides support for broad applicability of this model among ehrlichial pathogens.

Importantly, antibodies induced by outer membrane immunization, which results in high anti-MSP5 antibody titers that correlate with protection against homologous challenge (27), recognized the MSP5 immunodominant epitope in a conformationally dependent form, as shown by the results in Table 1. Furthermore, the polyclonal antibody induced by native MSP5 immunization also recognized predominantly conformationally dependent epitopes on the *A. marginale* surface. This indicates that the surface binding of antibody to all MSP5 immunodominant epitopes is conformationally dependent and is consistent with a requirement for native-protein secondary and tertiary structures in effective immunization.

In contrast to the secondary- and tertiary-structure requirements for MSP5 B-cell epitopes, the role of the quatenary structure remains unclear. Membrane MSP5 and MSP2 occur as both intramolecularly disulfide bonded monomers and intermolecularly disulfide linked multimers. Although monomeric MSPs, including MSP2 and MSP5, induce antibody against the *A. marginale* surface, complete neutralization of infectivity may require antibody directed against functional surface regions composed of two or more MSPs (13, 30). This possibility is suggested by the greater inhibition of *A. marginale* binding to the erythrocyte surface by antibodies generated against native organisms or a complex of MSP1a and MSP1b compared to antibody generated against MSP1a and MSP1b individually (9, 10). The importance of antibody against multiple MSPs is also supported by the complete neutralization of in vivo infectivity by antibody generated against the intact outer membranes (21). Whether intermolecular bonding of MSPs results in different B-cell epitopes from those resulting from intramolecularly bonded MSPs is unknown, although the high degree of conformational dependence shown in the present study suggests that changes in bonding pattern are likely to alter the surface-exposed epitopes. Consequently, defining the structural requirements of critical outer membrane epitopes is a priority and will support the development and testing of vaccines that maintain native MSP structure. These approaches include recombinant MSP immune system-stimulating complexes, expression of multiple recombinant MSPs in the outer membranes of live bacterial vectors, and direct immunization with DNA encoding MSPs.

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FUNCTION-DEPENDENT CONFORMATIONAL CHANGES OF THE ABCG2 MULTIDRUG TRANSPORTER MODIFY ITS INTERACTION WITH A MONOCLONAL ANTIBODY ON THE CELL SURFACE

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Abbreviations:

ABCG2: human MXR/BCRP/ABCP multidrug transporter; ABC transporters: ATP-Binding Cassette transporters; AMP-PNP: Adenosine 5'-(β,γ-imido)triphosphate; DFP: diisopropyl-fluorophosphate; FP: flavopiridol; GAM-PE: goat anti mouse phycoerythrin conjugated secondary antibody; mAb: monoclonal antibody; MDR1: human multidrug resistance protein (P-glycoprotein, ABCB1); MRP1: human multidrug resistance protein 1, ABCC1; MX: mitoxantrone; PFA: paraformaldehyde; Sf9 cells: *Spodoptera frugiperda* ovarian cells; V_i: sodium-orthovanadate.

ABSTRACT

The human ABCG2 protein is an important primary active transporter for hydrophobic compounds in several cell types, and its overexpression causes multidrug resistance in tumors. A monoclonal antibody (5D3) recognizes this protein on the cell surface. In ABCG2-expressing cells 5D3 antibody showed a saturable labeling and inhibited ABCG2 transport and ATPase function. However, at low antibody concentrations 5D3 binding to intact cells depended on the actual conformation of the ABCG2 protein. ATP depletion, or the addition of the ABCG2-inhibitor Ko143, significantly increased, while the vanadate-induced arrest of ABCG2 strongly decreased 5D3 binding. The binding of the 5D3 antibody to a non-functional ABCG2 catalytic center mutant (K86M) in intact cells was not affected by the addition of vanadate, while still increased by Ko143. In isolated membrane fragments the ligand modulation of 5D3 binding to ABCG2 could be analyzed in detail. In this case 5D3 binding was maximum in the presence of ATP, ADP or Ko143, while the non-hydrolysable ATP analog, AMP-PNP, and nucleotide trapping by vanadate, decreased antibody binding. In membranes, expressing the ABCG2-K86M mutant, both ATP, ADP and AMP-PNP decreased, while Ko143 increased 5D3 binding. Based on these data we suggest that the 5D3 antibody can be used as a sensitive tool to reveal intramolecular changes, reflecting ATP binding, the formation of a catalytic intermediate, or substrate inhibition within the transport cycle of the ABCG2 protein.

INTRODUCTION

The ABCG2 (MXR/BCRP/ABCP) protein causes multidrug resistance in cancer cells and may have an important function in physiological protection of various tissues against toxic agents. ABCG2 was first cloned from the placenta, where it is most abundantly expressed (1). The overexpression of ABCG2 was observed in certain drug-resistant cell lines and tumors, providing a special multidrug resistant phenotype in these cancer cells (2-5). The ABCG2 protein is a so-called ABC half-transporter, which has only one nucleotide binding (ABC) and one transmembrane domain, and most probably works as a homodimer in the plasma membrane (6-11).

The overexpression of ABCG2 was documented in several human tumors, which indicates its possible importance in the multidrug resistant phenotype of various cancer cells (12-15). The substrate specificity of ABCG2 partially overlaps with the other major multidrug resistance ABC transporters, MDR1 and MRP1, that is the compounds transported by ABCG2 are also large, hydrophobic molecules, including mitoxantrone, topotecan, flavopiridol, methotrexate and Hoechst 33342 (13,16,17).

ABCG2 was found to be physiologically expressed in the liver, small intestine, colon, lung, kidney, adrenal and sweat glands, and in the endothelia of veins and capillaries. The functional characteristics and the tissue distribution of ABCG2 suggest a major role in the tissue protection against xenobiotics (4,13,18). High level expression of the ABCG2 protein and its fluorescent dye extrusion function has been suggested for the identification of bone marrow stem cells (17). Moreover, this so called "side population" of progenitor cells, actively extruding the fluorescent Hoechst 33342 dye, seems to contain pluripotent stem cells in a variety of tissue sources (17,19-21).

The proper detection of the ABCG2 protein would be of major importance in cancer diagnostics, as well as in stem cell research and stem-cell based therapeutic developments. The recent development of a monoclonal antibody, specifically reacting with the human ABCG2 protein on the cell surface (17) has been a major breakthrough in this regard. This antibody was prepared by immunizing mice with intact mouse fibroblasts, expressing the human ABCG2. The antibody, named 5D3, was reported to inhibit the Hoechst 33342 dye transport function of ABCG2 in intact cells (22), and was made commercially available (eBioscience). Similar antibodies have already been prepared against the human MDR1 multidrug transporter (23,24). In the case of MDR1, several of the mAbs reacting with

extracellular epitopes were found to inhibit the transport function of the protein, and the reactivity of one of these antibodies, UIC2 was reported to depend on the conformation of the MDR1 protein (23,25-27).

In the present experiments we have studied the interaction of the anti-ABCG2 monoclonal antibody 5D3 in various cell types expressing the human ABCG2 protein, and examined the effects of ABCG2 protein modulators on this interaction. We have also compared these effects to those of cell fixation and/or permeabilization, and correlated ABCG2 protein detection with another monoclonal antibody, raised against an intracellular epitope of human ABCG2, BXP-21 (28). We also show here that 5D3 binding to ABCG2 in isolated membrane fragments can be analyzed, which allows a detailed investigation of the ligand modulation of antibody binding.

We found that the interaction of 5D3 with ABCG2 was strongly dependent on the modulation of the multidrug transporter protein, thus 5D3 binding to an extracellular ABCG2 epitope was conformation-sensitive. Based on these data, and on previous results for the interaction of human MDR1 protein with conformation-sensitive antibodies, we suggest a model for the transport cycle dependence of 5D3 antibody interaction with the ABCG2 protein. Our data indicate that this conformation-sensitive antibody interaction can be applied for studying the molecular mechanism and the detection of ligand interactions of ABCG2.

EXPERIMENTAL PROCEDURES

Materials

Mitoxantrone, Na-orthovanadate, propidium iodide, AMP, ADP, AMP-PNP and ATP were purchased from Sigma. Hoechst 33342 was purchased from Molecular Probes. BXP-21 antibody was obtained from Drs. George Scheffer and Rik Scheper (Department of Pathology Free University, Medical Center, Amsterdam, The Netherlands).

Cell lines and retroviral transduction

Retrovirus producing cells and HEK 293T cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The human PLB985 (in the following PLB) cells were kindly provided by Dr. M. Dinauer (Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, IN), the MCF-7 parental cells and the MCF-7/MX cells were gifts of Dr. Susan E. Bates (Cancer Therapeutics Branch, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, MD). PG13 (29) was obtained from the American Type Culture Collection, (Rockville, MD, USA). The construction of the ABCG2 retroviral vectors and cell transduction methods were described in detail in (30). Transduced cells in some cases were selected by stepwise increases in mitoxantrone or flavopiridol concentrations or single-cell cloned for the desired level of protein expression. Sf9 cells expressing the ABCG2 protein or its K86M variant were prepared as described previously (31). In the present study we used the K86M variant introduced into the wild type (R482) ABCG2, by cloning the Not1-Spe1 fragment of pAcUW21-L/K86M-R482G (31) into the corresponding site of the pAcUW21-L/R482 vector.

Immunodetection of ABCG2

For immunoblotting washed cells were suspended in the presence of 2 mM DFP in 2 × Laemmli buffer and sonicated for 3 × 5 seconds at 4°C. Sf9 membranes were also suspended in Laemmli buffer. The proteins separated on 7.5 % SDS-polyacrylamide gels were electroblotted onto PVDF membranes, and immuno-detection was performed by using the monoclonal antibody BXP-21 (500 × dilution), and a HRP-conjugated goat anti-mouse IgG (5,000 × dilution, Jackson Immunoresearch). Enhanced chemiluminescence (ECL) technique was applied to detect HRP activity on the blots.

For measuring ABCG2 expression by flow cytometry (Becton Dickinson FACS Calibur) 5D3 primary antibody (purified anti-human ABCG2, clone 5D3, e-Bioscience, Cat. No. 14-8888) or BXP-21 antibody and phycoerythrin-labeled anti-mouse secondary

antibody (GAM-PE, Beckman-Coulter) were used. 5D3 binding in intact cells was examined by suspending the cells in phenol red-free Hank's balanced salt solution with additional pH stabilization by 20 mM phosphate buffer. Aliquots of the suspension, containing 3×10^5 cells were incubated with 500 times diluted 5D3 primary antibody (1 μg/ml), 100 times diluted BXP-21 antibody, or mouse IgG2b (1 μg/ml, as isotype control) in 50 μl buffer for 45 minutes at 37°C (all labeling experiments were carried out in shaker water bath). After washing the cells with Hank's solution, containing 0.5% Bovine Serum Albumin (BSA), the cells were labeled by 200 times diluted goat anti mouse phycoerythrin conjugated secondary antibody (GAM-PE, 3 μg/ml), in 50μl buffer for 30 minutes at 37°C. After washing, the cells were resuspended in Hank's medium and 5D3 binding was determined at 488 nm excitation and 585/42 nm emission (FL2) wavelengths.

When the labeling was carried out with PFA-prefixed cells, the cells were incubated in 200 μl of PBS (phosphate buffered saline) solution containing 1% paraformaldehyde for 10 minutes at 37°C before the above mentioned labeling procedure.

For obtaining PFA-fixed and permeabilized cells, the cells were incubated in 200 μl PBS solution, containing 4% paraformaldehyde and 0.05% Triton-X 100, for 10 minutes at 37°C. The same 0.05% Triton-X 100 was present during all steps of the labeling procedure. When labeling was carried out in the presence of modifying agents (5 μM Ko143, 10 mM Na-orthovanadate, 50 μM flavopiridol or 5 μM mitoxantrone), the cells were preincubated with these agents for 10 minutes at 37°C before labeling, and the agents were present during antibody labeling. When applicable, ATP depletion of the cells was carried out before the labeling procedure by washing the cells twice in sugar-free Hank's medium and 30 minutes incubation at 37°C in Hank's medium containing 50 mM 2-deoxy-D-glucose and 15 mM sodium azide. During cell labeling and washing the media contained the same ATP-depleting agents.

Isolated membrane fragments from Sf9 cells (45 μg) were labeled with 1 μg/ml 5D3 (or mouse IgG2b as isotype control) in 100 μl final volume of assay mix (40 mM MOPS-Tris pH 7.0, 5 mM Na-azide, 50 mM KCl, 2 mM DTT and 500 μM EGTA-Tris pH 7.0) for 30 minutes at 37°C. The membranes were then washed with 500 μl assay mix and pelleted at 10,000 g for 4 minutes. The pellet was suspended in assay mix, containing 1 μg/ml GAM-PE, and incubated at 37°C for 30 minutes. The membranes were then washed and centrifuged (10,000 g for 4 minutes). Finally, the pellet was suspended in 200 μl assay mix

and the fluorescence was detected in a fluorescence plate reader (Fluoroskan II, Labsystems) at 485 nm (excitation)/ 590 nm (emission). When the effects of different agents were investigated the membranes were preincubated in assay mix containing 2 mM Na-orthovanadate, 1 μM Ko143, 10 mM MgAMP, MgADP, MgAMP-PNP, MgATP or 10 mM AMP, ADP, AMP-PNP, ATP + 2 mM EDTA or the combination of these agents (as described in the Figure Legends) for 5 minutes at 37°C prior to the addition of the 5D3 antibody. The relative level of 5D3 binding was calculated as follows: $(F_X-F_{IT})/(F_0-F_{IT})*100$. F_X: fluorescence measured in the presence of 5D3 and the investigated compound, F_{IT}: fluorescence measured in the presence of mouse IgG2b (isotype control), F_0: fluorescence measured in the presence of 5D3 alone.

Cellular mitoxantrone uptake

The drug extrusion function of ABCG2 in intact cells was evaluated by the mitoxantrone (MX) uptake assay of Robey et al. (32) as modified by (30). After 5D3 labeling at 37°C for 30 minutes and washing (as described for immuno-labeling), the cells were suspended in phenol red-free Hank's balanced salt solution containing 5 μM MX or 5 μM MX + 5 μM Ko143 (in some experiments 10 mM Na-orthovanadate, or 50 μM flavopiridol) and incubated at 37°C for 30 minutes. After washing, MX fluorescence was analyzed by flow cytometry (FACSCalibur, Becton Dickinson) at 635 nm excitation and 661/16 nm emission wavelengths (FL4). Dead cells were excluded based on propidium iodide (5 μg/ ml) staining.

Measurement of Hoechst 33342 transport activity

Accumulation of Hoechst dye (Hst) was performed by using intact PLB-ABCG2 (R482), PLB-MDR1 or parental PLB cells (30) in a fluorescence spectrophotometer (Perkin Elmer LS 50B) at 350 nm (excitation)/ 460 nm (emission). The cells (3×10^5) were incubated with or without 12 μg 5D3 antibody in 100 μl final volume of the transport buffer (120 mM NaCl, 5 mM KCl, 400 μM $MgCl_2$, 40 μM $CaCl_2$, 10 mM HEPES, 10 mM $NaHCO_3$, 10 mM glucose and 5 mM Na_2HPO_4) 37°C for 30 minutes. Hoechst transport was then determined on 5D3 labeled or non-labeled cells, as described (33).

ATPase activity measurement

Sf9 membranes containing human ABCG2, MDR1 or ABCG2-K86M were harvested and their membranes were isolated and stored at -80 °C according to (34,35). ATPase activity was measured as described previously, by determining the liberation of inorganic

phosphate from ATP with a colorimetric reaction (11). When the effect of antibody binding was investigated, membranes were preincubated with anti-ABCG2 5D3 monoclonal antibody (eBioscience) or mouse IgG2b (isotype control, SIGMA) in 20 or 160 μg/ mg membrane concentration for 30 minutes at 37°C and then washed twice in ice-cold buffer (40 mM MOPS-Tris pH 7.0, 50 mM KCl, 2 mM dithiotreitol and 0.5 mM EDTA) prior to the ATPase activity measurement. The figures represent the mean values of at least three independent experiments with duplicates.

RESULTS

Antibody detection of ABCG2

For the immuno-detection of the human ABCG2 protein in various cell types we used two monoclonal antibodies. The BXP-21 antibody was generated against an N-terminal intracellular epitope (aa. 271-396 - see (28)), while mAb 5D3 was produced by immunizing mice with intact mouse fibroblasts expressing the human ABCG2 protein (17). As documented earlier, BXP-21 recognizes the ABCG2 protein both in immunoblots and in permeabilized cells (28). In contrast, the 5D3 antibody could be used to recognize human ABCG2 on the surface of intact cells (17), but not on immunoblots (see below).

Fig. 1 shows immunoblot detection of the human ABCG2 protein in the various cells used in the present study, by mAb BXP-21. Panel A shows expression of human, wild-type ABCG2 or the K86M-ABCG2 variant in isolated membranes of Sf9 insect cells (11).

Panel B shows BXP-21 immunoreactions with cell lysates of PLB cells, engineered to express the wild-type ABCG2 or its K86M mutant variant. The expression level of the K86M variant of ABCG2 was about one third of the expression obtained for the wild-type protein (these cells could not be selected by mitoxantrone – see Experimental procedures and (31)).

Fig. 1, Panel C documents the retrovirally evoked expression of human ABCG2 in HEK-293T cells, and Panel D demonstrates the overexpression of ABCG2 in the mitoxantrone-selected MCF-7 cell derivative (MCF-7/MX), as detected by the BXP-21 antibody. It should be noted that, in accordance with previous results, we did not find any immuno-reactivity of the 5D3 antibody with ABCG2 on immunoblots.

Fig. 2 demonstrates the detection of ABCG2 in the parental and the ABCG2-expressing PLB cells, respectively, by flow cytometry and using the BXP-21 and the 5D3 monoclonal antibodies. In these experiments, each antibody was used in a concentration of 0.2 $\mu g/10^6$ cells.

We found that in the parental PLBs the 5D3 antibody showed no immunoreactivity, even if the cells were fixed by PFA, or fixed and permeabilized by PFA+Triton X-100 treatment (Fig. 2, Panel A). When parental PLB cells were labeled with the BXP-21 antibody (Fig. 2, Panel B), there was some background labeling observed, as compared to the isotype control. However, in these parental cells BXP-21 labeling did not increase upon treatment with PFA or PFA+Triton X-100.

As shown in Figure 2, Panel D, in the case of the ABCG2-expressing PLB cells, there was no reaction with the BXP-21 mAb, unless the cells were both fixed and Triton-permeabilized. In this latter case a significant, ABCG2-dependent labeling of the cells by BXP-21 was found. In contrast, the 5D3 antibody showed a well visible immunoreactivity with the native ABCG2-expressing PLBs (Figure 2, Panel C). This reactivity was increased by PFA fixation, while a further permeabilization with Triton X-100 had no effect on 5D3 binding.

It has to be noted that a similar shift in 5D3 reactivity was found upon PFA fixation, and independent of membrane permeabilization, in all ABCG2 expressing cell types studied, including Sf9 insect cells (not shown here). The 5D3 labeling in this latter cell line indicates that the level or even the absence of N-glycosylation does not influence the interaction of 5D3 antibody with ABCG2.

Inhibition of ABCG2 function by the 5D3 antibody

The data presented in Fig. 2 were obtained with relatively low concentrations of the 5D3 antibody (0.2 μg/ 10^6 cells). By increasing the antibody concentration up to 10 μg/10^6 cells, a saturable level of ABCG2 labeling could be achieved, which was not significantly modified by PFA fixation (Figure 3A).

In order to investigate the effect of 5D3 on the ABCG2 function, we preincubated the PLB-ABCG2 cells with the 5D3 antibody (40 μg/10^6 cells) and then measured Hoechst 33342 dye extrusion. As shown in Fig. 3B, at high 5D3 concentrations (40 μg/10^6 cells), a significant ($p = 0.002$), about 65% inhibition of dye transport was observed. In contrast, 5D3 did not inhibit the Hoechst dye transport measured in MDR1-expressing PLBs. In addition, the anti-MDR1 inhibitory monoclonal antibody, UIC2 inhibited Hoechst 33342 extrusion in the MDR1-expressing cells, while did not modify the transport activity in the PLB-ABCG2 cells (not shown).

In order to further explore the ABCG2 inhibitory potential and selectivity of the 5D3 antibody, we have performed direct ABCG2-ATPase measurements in isolated Sf9 cell membranes (Fig. 3C). In these experiments we preincubated the isolated membranes for 30 min at 37°C with two different 5D3 concentrations (20 μg and 160 μg 5D3/ mg membrane protein, respectively) in the absence of ATP, to assure maximum 5D3 labeling of ABCG2 (see below). We found that the application of the lower, 20 μg/mg membrane 5D3 concentration, although at least 20 times greater than that used in the whole-cell experiments, did not significantly affect the ABCG2-ATPase ($p = 0.1$). However, when the

ATPase activity was measured after labeling with 160 μg 5D3/mg membrane protein, a significant ($p= 0.007$), about 30% decrease in the vanadate-sensitive ATPase activity of ABCG2 was observed. No inhibition was seen in the presence of similar concentrations of an isotype control antibody. There was no effect of 5D3 antibody on the ATPase activity of MDR1 or ABCG2-K86M membranes. All these data indicate that the 5D3 antibody, when applied in high concentrations, specifically inhibits the transport and ATPase function of the ABCG2 protein.

Effects of ABCG2 inhibitors on 5D3 reactivity and mitoxantrone transport by ABCG2 in intact cells

In the following experiments we have studied the effects of a specific ABCG2 inhibitor, Ko143 (36) and the general ABC transporter inhibitor, Na-orthovanadate (V_i) on the binding of 5D3 antibody in intact cells by flow cytometry. The 5D3 labeling conditions were as described for Fig. 2, that is relatively low antibody concentrations were applied. In the same cells we have also measured mitoxantrone (MX) accumulation, by using a different fluorescence detection channel (see Experimental procedures).

As shown in Fig. 4, Panel A, in the parental PLB cells 5D3 reactivity was negligible, and unchanged by the addition of Ko143 or Na-orthovanadate (V_i). MX accumulation in the same cells reached a high level and was unaffected by the presence of Ko143 or V_i (Fig. 4, Panel B).

In the ABCG2-expressing PLBs we found a low, but measurable 5D3 reactivity (Fig. 4. Panel C), which was greatly increased by Ko143, while slightly reduced by the addition of Na-orthovanadate. In the parallel MX uptake experiments (Panel D), in the ABCG2-expressing PLBs MX accumulation was reduced, as compared to that found in the parental cells. ABCG2 inhibition by both Ko143 and V_i significantly increased intracellular MX level, similar to that seen in cells not expressing ABCG2. Cell labeling with 5D3 at these low antibody concentrations did not cause any change in MX uptake.

According to these results, both Ko143 and V_i blocked the ABCG2 transporter function, but Ko143 increased, while V_i rather decreased 5D3 binding on the cell surface. On the other hand, 5D3 labeling at this lower antibody concentrations did not inhibit MX transport activity of ABCG2 (see below).

When we analyzed 5D3 binding and MX uptake in other ABCG2 expressing mammalian cell types, we found a similar modulation of 5D3 binding and MX transport by these inhibitors. The data presented in Fig. 4, Panel G, document that in ABCG2-

transduced HEK-293T cells 5D3 binding was decreased by V_i treatment and increased by Ko143. As shown in Panel H, MX transport in these cells was inhibited by both inhibitors (interestingly, vanadate preincubation could not block MX extrusion in all HEK cells, a variable population of transporting cells was still observed in these experiments). Parental HEK cells did not show a significant ABCG2 expression or MX transport activity (Panels E and F).

We obtained essentially similar data in the MCF-7/MX cells and the PLBs expressing the gain-of function R482G mutant of ABCG2 (not shown). As a summary, the addition of Ko143 and V_i treatment blocked ABCG2 function in all these cell types, and Ko143 significantly increased, while Na-orthovanadate decreased 5D3 binding to the ABCG2 protein.

Effect of ATP depletion and transported substrates on 5D3 reactivity and mitoxantrone transport by ABCG2 in intact cells

In the following experiments we have studied the effect of ATP depletion and various transported substrates on 5D3 binding and MX extrusion by ABCG2 in intact PLB cells. For achieving an efficient ATP depletion of the ABCG2-expressing PLBs, we used a 30 min pretreatment at 37°C, with a combination of Na-azide and 2-deoxy-D-glucose (see Experimental procedures). As documented earlier in many hematopoietic cell lines, this treatment reduces the ATP level below 5% of the original levels and results in the accumulation of both ADP and AMP in the cells.

As shown in Fig. 5, this ATP depletion strongly inhibited the ABCG2 transport function, that is eliminated the ABCG2-dependent MX extrusion in these cells (Panel B). Interestingly, ATP depletion significantly increased 5D3 binding, thus transforming the ABCG2 protein in a conformation optimal for 5D3 labeling (Panel A).

We have examined the effects of various agents on 5D3 binding, which were demonstrated transported substrates of the ABCG2 protein. The co-incubation of the ABCG2 cells with mitoxantrone (2-5 μM) did not influence 5D3 labeling (see Figure 5 Panel C). We also found no appreciable effect on 5D3 binding by the addition of other substrates, prazosin (10-50 μM), or ZD1839 (0.1-1 μM) (not shown) (5,32,33). Flavopiridol (FP), another transported substrate of ABCG2 (37) in low (1-5 μM) concentrations had no effect on 5D3 antibody labeling, while in concentrations above 50 μM this agent significantly increased 5D3 labeling and interfered with MX extrusion (Figure 5 Panels B and C). This is in line with the ABCG2-ATPase measurements, where

high flavopiridol concentrations were inhibitory, thus could act similarly to Ko143 (data not shown in detail).

Effects of substrates, inhibitors and ATP depletion on 5D3 reactivity in the mutant, non-functional K86M-ABCG2, expressed in intact cells

In the next set of experiments we studied intact mammalian cells expressing a non-functional mutant (K86M) variant of ABCG2. This mutation in the highly conserved Walker A motif does not affect ATP binding by ABCG2, but impairs its drug transport and ATPase activity, as well as the formation of a vanadate-induced trapped nucleotide (31). As shown in Fig. 6, Panels A and B, this K86M-ABCG2 had no MX extrusion function, but showed a well measurable 5D3 binding on the cell surface.

In these studies we found that the 5D3 binding of the K86M mutant ABCG2 was significantly increased by PFA fixation, ATP-depletion or Ko143 treatment. Still, the relative increase in 5D3 binding due to these effects was much smaller than in the case of the wt ABCG2, and 5D3 binding was unaffected by pretreatment with Na-orthovanadate (Fig. 6. Panel A). Thus the non-functional K86M variant of ABCG2 showed a relatively high 5D3 binding in its native state, but in the case of ATP-removal and Ko143 treatment similar conformational changes were detected by 5D3 in this mutant variant as in the wild-type protein. The lack of the formation of a transition-state intermediate in the K86M-ABCG2 correlated with the absence of an effect of Na-orthovanadate.

Effects of nucleotides and transport inhibitors on 5D3 reactivity of ABCG2 in isolated membrane fragments

In the following experiments we examined the effects of various nucleotides and transport inhibitors on 5D3 binding by human ABCG2 and its mutant (K86M) variant in isolated insect cell membrane fragments. In these membrane preparations ABCG2 expression reaches a high level (up to 5% of the membrane proteins), in a fully active form, as reflected by the ABCG2-ATPase activity (11,31). A large fraction of the isolated membrane fragments are accessible both from the cytoplasmic and the external cell surface, as tested by the trypsin sensitivity of open fragments (38) and simultaneous staining of the membrane fragments with two antibodies (pAb 405 and mAb 5D3), that recognize an intracellular (5), and an extracellular epitope of ABCG2, respectively (not shown here). Therefore this assay system allows a direct estimation of the effects of cytoplasmic ligands on the cell surface interaction of ABCG2 with the 5D3 antibody.

As shown in Fig. 7, Panel A, 5D3 binding to isolated Sf9 cell membranes, containing the human ABCG2 protein, reached a high level, significantly exceeding that seen in the control, MDR1-containing membranes, or the labeling obtained with an isotype control antibody.

Fig 7 B and C document the effects of various ligands on 5D3 binding to wild-type (Panel B) or K86M (Panel C) ABCG2 in isolated membranes. In the case of the wild-type ABCG2 (Panel B), the addition of MgAMP, MgADP, or MgATP did not significantly modulate the level of 5D3 labeling, while MgAMP-PNP, a non-hydrolysable ATP analog greatly reduced 5D3 binding. The addition of Na-orthovanadate was ineffective in the presence of MgAMP, while produced a major decrease in 5D3 binding together with MgATP. When the cells were preincubated with the transport inhibitor Ko143, either in the presence of MgATP or MgAMP-PNP, a maximum level of 5D3 binding to ABCG2 was observed. Ko143 preincubation produced a maximum 5D3 binding even in the presence of MgATP+vanadate. An interesting finding was in these experiments, that if Ko143 was added after a preincubation with MgAMP-PNP, the reduction in 5D3 binding by this nucleotide could not be reversed by Ko143 (data not shown).

These data indicate that in the case of a functional ABCG2, 5D3 labeling has a relatively high level either in a nucleotide-free, or in a nucleotide-liganded, flexible state of the transporter. However, when the transport cycle is blocked by a non-hydrolysable ATP analog, or by the inhibition of ATP hydrolysis by Na-orthovanadate, a strong reduction in

5D3 binding occurs. Arresting the ABCG2 transport cycle by Ko143, however, produces a high 5D3 binding, and this effect is not reversed the nucleotides and/or vanadate. Still, a low 5D3 binding conformation first fixed by MgAMP-PNP, cannot be changed to a high binding form by a later addition of Ko143.

Fig. 7, Panel C shows 5D3 binding in isolated membranes containing the K86M, non-functional mutant ABCG2 protein. In this case MgAMP had no effect, while both MgATP, MgADP and MgAMP-PNP significantly reduced 5D3 labeling. Na orthovanadate did not modify 5D3 binding, as compared to that seen with the respective nucleotides (MgAMP or MgATP). The addition of Ko143, again even in the presence of MgATP, MgADP, or MgAMP-PNP, produced maximum 5D3 binding.

These data can be interpreted to mean, that while MgAMP does not show binding to the protein, both MgATP, MgADP and MgAMP-PNP are bound to K86M-ABCG2 and, in the absence of a full catalytic cycle, they fix the transporter in a nucleotide-bound, reduced 5D3 binding state. This fixation does not require the presence of vanadate. These findings are in agreement with the unchanged ATP binding, but the lack of vanadate-dependent nucleotide trapping in the case of this mutant protein (31). Interestingly, Ko143 can still stabilize the K86M-ABCG2 variant in a high 5D3 binding state.

In experiments not documented here in detail, we have performed 5D3 binding to ABCG2 in isolated Sf9 membranes at 4°C, in order to investigate labeling at non-hydrolytic conditions. We found that 5D3 binding at 4°C was somewhat reduced (75 ± 1.4% of that measured at 37°C), and the addition of nucleotides or inhibitors (Ko143 or V_i) did not cause a measurable change in 5D3 binding.

We have also investigated 5D3 binding to ABCG2 in isolated Sf9 membranes upon the addition of AMP, ADP, AMP-PNP and ATP, but in the absence of Mg^{2+} ions (that is in the presence of excess EDTA), at 37°C. Interestingly, we found that in the absence of Mg^{2+}, both ADP, AMP-PNP and ATP (but not AMP) significantly decreased 5D3 binding to the ABCG2 protein. These effects were similar both in the wild-type ABCG2 and the K86M mutant variant (not documented in detail). These data indicate that the binding of ADP, ATP or AMP-PNP to ABCG2 (causing low 5D3 reactivity) occurs even in the absence of Mg^{2+}, but no further steps of the catalytic cycle are performed.

DISCUSSION

In the present experiments we have studied the interaction of the 5D3 monoclonal antibody, prepared against a cell surface epitope of human ABCG2, with this multidrug transporter both in intact cells and in isolated membranes. We found that in intact cells 5D3 recognition of the ABCG2 protein occurred at an external epitope. The specific antibody binding was significantly increased by fixation of the intact cells by paraformaldehyde (PFA), but this interaction did not require membrane permeabilization (Figure 2 Panel C). In contrast, the interaction of BXP-21 (an antibody raised against an intracellular epitope) with ABCG2 entirely depended on permeabilization of the cell membranes, making the intracellular epitopes accessible for this antibody (Figure 2 Panel D).

In accordance with data in the literature regarding 5D3 effect on ABCG2-induced drug resistance (22), we found that the 5D3 antibody significantly inhibited both the dye transport and the ATPase activity of the ABCG2 protein (Figure 3, Panels B and C). Still, the inhibition of the transport or ATPase activity of ABCG2 found here was incomplete even at very high 5D3 concentrations (see Figures 3B and 3C). This finding is most probably due to the steric and mechanical constrains in such antibody-transporter interactions. A similarly selective, but only partial functional inhibition has been reported for several anti-MDR1 antibodies, e.g. MRK16 or UIC2, reacting with cell surface epitopes of the MDR1 multidrug transporter (23,24).

In this study we found that at low 5D3 concentrations the actual conformation of the ABCG2 protein significantly modified 5D3 binding to the extracellular epitope. In intact cells ABCG2 interaction with 5D3 was greatly increased by the inhibition of ABCG2 function with a specific, high affinity inhibitor, Ko143 (see Figure 4, Panels C and G), or by cellular ATP depletion. (Fig. 5, Panel A). Similarly, an increase in 5D3 reactivity was observed in the presence of high, inhibitory concentrations of a drug substrate of ABCG2, flavopiridol (Figure 5C) (37).

In contrast, a reduction in 5D3 binding was observed when the cells were preincubated with Na-orthovanadate, a transition-state inhibitor of ABC transporters, including ABCG2 (31,39-41). In this case, within the nucleotide binding domain of the protein, vanadate anions replace phosphate after ATP hydrolysis, and the transport cycle of ABCG2 is arrested in a transition-state. This can be experimentally followed by measuring the

vanadate-dependent trapping of MgADP within the protein, which becomes incapable for further ATPase or transport activity (39-41). In the present experiments the arrest of the ABCG2 transport cycle by Ko143, by the removal of the energy donor substrate, ATP, as well as by Na-orthovanadate was documented by the lack of active mitoxantrone (MX) extrusion in the same cells (Figure 4, Panels D and H, Figure 5, Panel B). In these experiments the addition of low concentrations of transported substrates did not significantly modify cell surface 5D3 binding to ABCG2 (Figure 5, Panel C).

According to these data, 5D3 interaction with ABCG2 in intact cells depends on the actual conformation within the transport cycle of this multidrug resistance protein. 5D3 binding is relatively low in the case of the actively functioning protein or in its stabilized transition state. In contrast, 5D3 binding is greatly increased when ABCG2 conformation is stabilized in other specific conformations (by Ko143 or ATP depletion). In unpublished experiments we found that Ko143 inhibition of ABCG2 was reversible by repeated washings. Also, ABCG2-ATPase inhibition achieved by low (10 nM) Ko143 concentration could be removed by the addition of increasing concentrations of transported substrates, e.g. prazosin. These results indicate that Ko143 probably inhibits ABCG2 by interacting with its substrate binding site.

When examining the binding of the 5D3 antibody in intact cells to a non-functional ABCG2 catalytic center mutant (K86M-ABCG2), we found that 5D3 binding to this mutant protein was also efficient. In the case of this mutant ABCG2, 5D3 binding was not affected by the addition of transported substrates or vanadate, while it was increased by ATP-depletion or by the addition of Ko143 (Figure 6). These data are in line with the impaired catalytic cycle and transition state forming ability of this mutant, with unchanged ATP binding (31), and probably with conserved drug/inhibitor binding properties.

In order to further explore the mechanistic details of the ABCG2 catalytic cycle, we have performed a detailed analysis of 5D3 binding to ABCG2 in isolated membrane fragments, accessible from both sides of the membrane (see Fig. 7, Panel B). It is important to note that the experiments carried out with isolated membranes exclude the possibility that the changes in 5D3-ABCG2 interactions might be due to variable cell surface expression of the multidrug resistance protein in intact cells. They also allow to study the interaction of non cell-permeating ligands with cytoplasmic domains of the transporter.

In these experiments we observed that the non-hydrolysable ATP analog, AMP-PNP, strongly reduced 5D3 binding to ABCG2. MgAMP, MgADP, or MgATP had no major effect, but MgATP+Na-orthovanadate induced a major decrease in 5D3 binding. Preincubation with the inhibitor molecule, Ko143 maximized 5D3 binding under all conditions.

In the K86M-ABCG2 variant, the addition of MgATP, MgADP and MgAMP-PNP, all caused a major reduction of 5D3 binding, which was not further modulated by Na-orthovanadate. These results coincide with the conserved ATP binding, but impaired catalytic intermediate formation by this mutant protein. In the case of this non-functional mutant we still found an increased 5D3 binding upon preincubation with Ko143, even if MgATP, MgADP or MgAMP-PNP were added thereafter to the media (see Fig. 7, Panel C). These results suggest a preserved substrate/inhibitor binding site in this mutant protein.

Interestingly, in Sf9 membranes containing either wild-type ABCG2 or its K86M mutant, in the absence of Mg^{2+} (that is in the presence of EDTA), both ATP, ADP and AMP-PNP caused a decrease in 5D3 binding (not shown in detail). These data may indicate that at these high nucleotide concentrations (10 mM), ABCG2 binds nucleotides even in the absence of Mg^{2+}, although further ATP hydrolysis is absent.

When trying to investigate the possible effects of transported substrates (e.g. prazosin, flavopiridol, or mitoxantrone) on 5D3 binding by ABCG2 in isolated Sf9 cell membrane fragments, we could not detect any major changes evoked by relevant substrate concentrations. This is similar to the lack on ATPase stimulation by substrates in this system, and most probably due to the presence of endogenous substrates of ABCG2 in the Sf9 membranes (31).

These data collectively indicate that the binding of the 5D3 monoclonal antibody closely reflects the changes in the drug- and ATP binding, as well as the catalytic state of the ABCG2 transporter. This is most probably due to the variable appearance of a conformational epitope within the ABCG2 protein on the cell surface. This study is the first demonstration of such a conformation-sensitivity of an antibody binding to the ABCG2 protein, although a conformation dependent binding of some extracellular antibodies, e.g. MRK16 or UIC2, to another multidrug transporter, the MDR1 protein, has already been documented (23,24). The determination of the actual epitope structure involved in 5D3 binding should require a detailed molecular mapping of potentially cell-surface domains of ABCG2.

As a summary, the various steps within the catalytic cycle of the ABCG2 multidrug resistance transporter could be visualized through changes in 5D3 binding. A low level 5D3 binding was observed when the non-hydrolysable ATP analog, MgAMP-PNP, or the addition of ATP or ADP without Mg^{2+} ions stabilized the protein in a pre-hydrolytic state (42,43). The formation of a catalytic intermediate, reflected by nucleotide trapping in the presence of vanadate anions (40,41), also coincided with a low 5D3 reactivity of ABCG2. In contrast, transport inhibition by Ko143 or by high concentrations of flavopiridol, as well as by ATP depletion, stabilized the protein in a conformation with high 5D3 binding capacity.

Based on these data we suggest that the 5D3 reactive form of ABCG2 is a stabilized "substrate off-site" conformation of the transporter. It has to be noted that the ABCG2 protein is an ABC half-transporter, and its function requires homo-dimerization (6-8,11). Conformational changes detected through a complex extracellular epitope of a membrane protein can be due to a function-dependent rearrangement of the transmembrane helices, triggering the movements of the extracellular loops, or to the surface-exposure of membrane-embedded short segments (for MDR1 see (44)). ABCG2 acts as a homodimer, and one additional possible explanation for the conformational changes described in the present study is the function-dependent re-orientation of the monomers within the dimer, or facilitation of the dimer formation. However, further experiments are needed to elucidate the dependence of 5D3 binding on the molecular interactions between the dimerizing ABCG2 molecules.

Based on this study we suggest that the 5D3 antibody can be used to reveal major intramolecular changes in the ABCG2 protein during its catalytic/transport cycle. Examining 5D3 binding to various mutant, polymorphic, or stabilized forms of ABCG2 may further help structure-function relationship studies. Moreover, based on the present data, optimum conditions can be selected for the investigation of ABCG2 expression and function by 5D3 binding in intact cell preparations, thus employing this antibody for a sensitive clinical laboratory detection of ABCG2 expression and function.

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LEGENDS FOR THE FIGURES

Figure 1. Immunoblot detection of human ABCG2 by monoclonal antibody, BXP-21.

Sf9 membranes containing wtABCG2, ABCG2-K86M or β-galactosidase (Panel A) and cell lysates from PLB (Panel B), HEK 293 (Panel C) and MCF-7/MX (Panel D) cells expressing wtABCG2 (or ABCG2-K86M) or parental cells (ctr.) were subjected to Laemmli gel electrophoresis and electroblotting. The amount of protein samples loaded on the gel were 2 μg for Sf9 membranes, HEK and MCF-7 cells, and 40 μg for PLB cells lysates. ABCG2 was detected by the BXP-21 monoclonal antibody. Experiments were performed three times, the Figure shows the result of one representative experiment.

Figure 2. Flow cytometry detection of ABCG2 in the parental PLB (Panel A and B), and the ABCG2-expressing PLB cells (Panel C and D), by the 5D3 and the BXP-21 monoclonal antibodies. Effect of fixation by PFA and permeabilization by PFA+Triton X-100.

PLB cells were treated with 1 % PFA (dashed line) or 4 % PFA + 0.05% Triton-X 100 (heavy solid line) prior to 5D3 (left panels) or BXP-21 (right panels) labeling. Non-treated PLB cells (native, solid line) were also labeled with one of the monoclonal antibodies or isotype control (IT, dotted line). Fluorescence of phycoerythrin conjugated secondary antibody was analyzed by flow cytometry (FACSCalibur, Becton Dickinson). Figure shows the result of one representative experiment.

Figure 3. Effect of 5D3 antibody concentration on the labeling (Panel A) or function of ABCG2 (Panels B and C).

Panel A: Labeling of PLB cells by 5D3.

PLB parental (left) or wtABCG2 expressing (right) cells were incubated with different concentrations of the 5D3 antibody: 0.2 μg 5D3/ 10^6 cells (L 5D3, solid line) or 10 μg 5D3/ 10^6 cells (H 5D3, dotted line) or isotype control (IT, dotted line). Cells fixed with 1 % PFA were also labeled with 0.2 μg (L 5D3+PFA, heavy solid line) or 10 μg (H 5D3+PFA, dashed line) / 10^6 cells 5D3 concentrations. Fluorescence of phycoerythrin conjugated secondary antibody was analyzed by flow cytometry (FACSCalibur, Becton Dickinson).

Panel B: Inhibition of the Hoechst 33342 dye transport by the 5D3 antibody in PLB cells.

3 x 10^5 PLB cells expressing wtABCG2 or MDR1 and control cells were incubated with (5D3, black columns) or without (control, white columns) 12 μg 5D3 and then Hoechst transport activity was measured in a fluorescence spectrophotometer (Perkin Elmer LS 50B) at 350 nm (excitation)/ 460 nm (emission). Hoechst transport was determined as described in Experimental procedures.

Panel C: Inhibition of the ABCG2-ATPase activity in isolated Sf9 cell membranes by the 5D3 antibody.

Sf9 membranes containing wtABCG2, ABCG2-K86M or MDR1 were incubated with 20 (low 5D3, hatched columns) or 160 μg (high 5D3, black columns) / mg membrane concentration of 5D3 antibody. ATPase activity was determined in 5D3 labeled or non-labeled (control, white columns) membranes by measuring vanadate sensitive inorganic phosphate liberation by colorimetric detection of inorganic phosphate liberation. Data points represent the mean ± standard deviation (S.D.) values of at least four measurements.

Figure 4. Flow cytometry detection of 5D3 mAb binding and mitoxantrone (MX) extrusion by ABCG2 in intact cells. Effects of Ko143 and Na-orthovanadate.

Panels A and B show parental PLB cells, Panels C and D represent ABCG2-expressing PLBs. Panels E and F show parental HEK293 cells, G and H show HEK293 cells expressing ABCG2.

Cells were incubated with 0.2 μg 5D3 antibody / 10^6 cells without (5D3, solid line) or with the addition of 5 μM Ko143 (dashed line) or 2 mM Na-orthovanadate (Vi, heavy solid line). IT means isotype control (dotted line). Mitoxantrone (MX) accumulation was measured on 5D3-labeled cells in the absence (5D3, solid line) or presence of 5 μM Ko143 (dashed line) or 2 mM Na-orthovanadate (heavy solid line). Experiments were performed three-times. Figure shows the result of one representative experiment.

Figure 5. Effects of ATP depletion and transported substrates on 5D3 binding (Panel A and C) and MX extrusion (Panel B) in ABCG2-expressing intact PLB cells.

PLB-ABCG2 cells were incubated in medium containing 50 mM 2-deoxy-D-glucose and 15 mM sodium azide (ATP depl., heavy solid line), 5 μM Ko143 (dashed line), 50 μM flavopiridol (FP, heavy solid line on Panel C or dotted line on Panel B) or 5 μM mitoxantrone (MX, dashed line) during the 5D3 labeling (Panels A and C) or MX

accumulation assay (Panel B). IT means isotype control. Experiments were performed three-times. Figure shows the result of one representative experiment.

Figure 6. Flow cytometry detection of the K86M-ABCG2 protein. 5D3 mAb binding (Panel A) and MX extrusion (Panel B) in K86M mutant ABCG2-expressing intact PLB cells. Effects of Ko143, ATP-depletion and Na-orthovanadate.

Cells were incubated with 0.2 μg 5D3 antibody / 10^6 cells without (5D3, solid line) or with the addition of 5 μM Ko143 (dashed line), 2 mM Na-orthovanadate (Vi, dashed line) or 50 mM 2-deoxy-D-glucose and 15 mM sodium azide (ATP depl., heavy solid line).

Figure 7. Detection of 5D3 mAb binding to ABCG2 in isolated Sf9 membrane fragments. Effects of nucleotides, Ko143, and Na-orthovanadate.

Panel A: Comparison of 5D3 and isotype control (IT) antibody binding to isolated Sf9 cell membranes. Isolated membrane fragments (45 μg) from Sf9 cells containing wtABCG2, ABCG2-K86M or MDR1 were labeled with 1 μg/ml 5D3 (black columns) or 1 μg/ml mouse IgG2b as isotype control (white columns). Fluorescence was detected in a fluorescence plate reader (Fluoroskan II, Labsystems) at 485 nm (excitation)/ 590 nm (emission).

Panel B: 5D3 binding to membranes containing wild-type ABCG2,

Panel C: 5D3 binding to membranes containing K86M mutant ABCG2.

Sf9 membranes containing wild-type ABCG2 (Panel B) or K86M mutant ABCG2 (Panels C) were incubated with 5D3 antibody in the presence of 10 mM MgAMP, MgADP, MgATP, MgAMP-PNP, MgAMP + 2 mM vanadate, MgATP + 2 mM vanadate, MgATP + 2 mM vanadate + 1 μM Ko143, MgADP + 1 μM Ko143, MgATP + 1 μM Ko143 or 10 mM MgAMP-PNP + 1 μM Ko143. When Ko143 was present, the membranes were preincubated with this inhibitor for 5 min before the addition of other reagents.

5D3 binding is shown in the percent fluorescence measured in the presence of 5D3 alone (see Experimental procedures).

Values shown are means of at least four independent experiments ± standard deviation (S.D.) values.

Figure 1

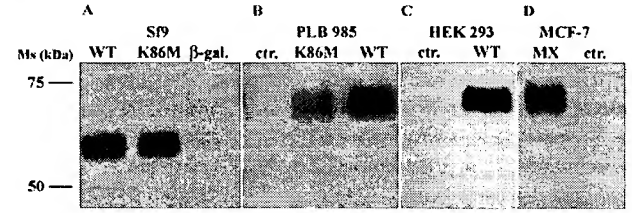


Figure 2

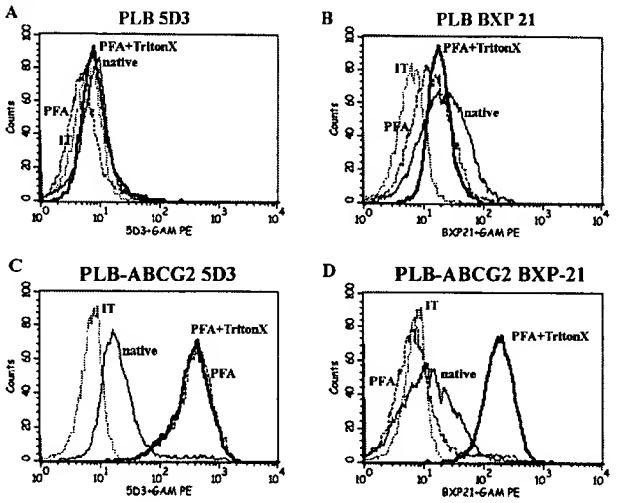


Figure 3

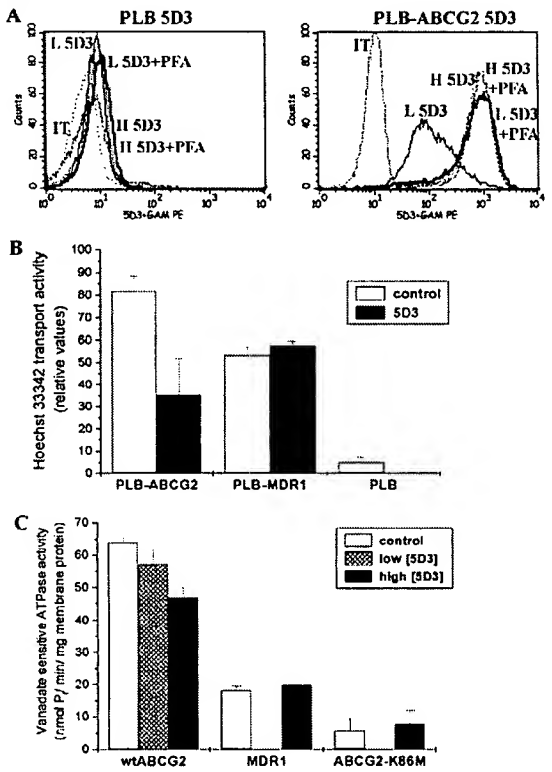


Figure 4

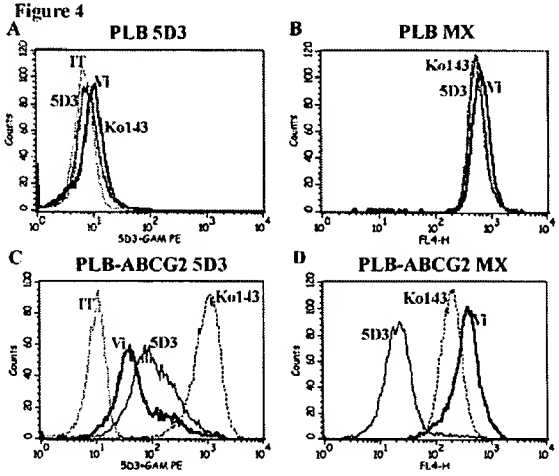


Figure 4

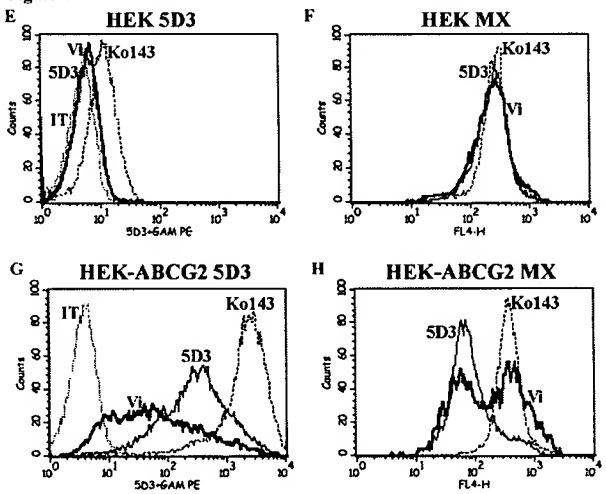


Figure 5

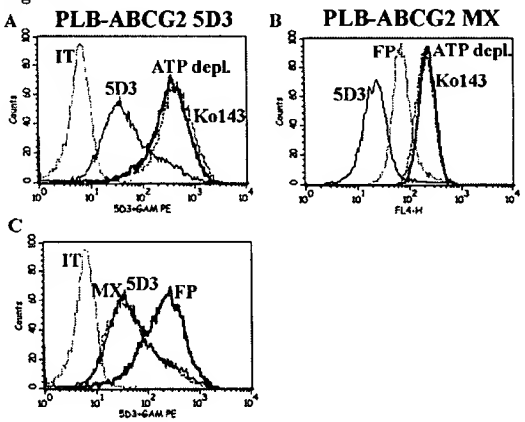


Figure 6

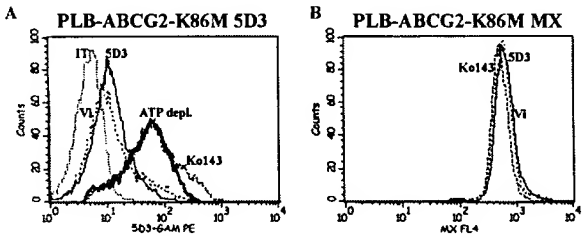
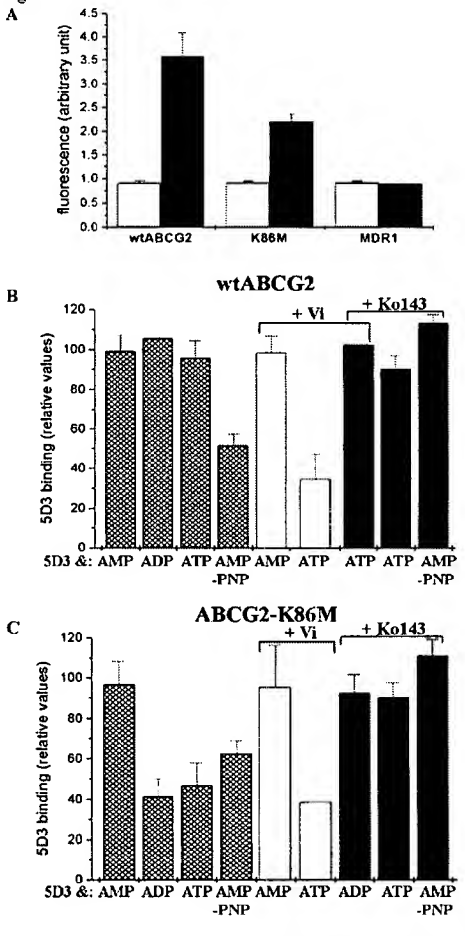
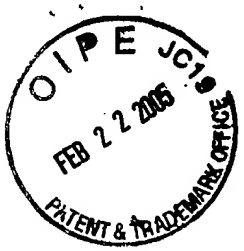


Figure 7





IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket No.:	1340-1-021CIP2 (SJ-0015)
Inventors:	Sorrentino and Schuetz
Serial No.:	09/866,866
Filing Date:	May 29, 2001
Examiner:	Li, Qian Janice
Customer No.:	31949
Group Art Unit:	1632
Confirmation No.:	4688
Title:	Method of Identifying and/or Isolating Stem Cells and Prognosing Responsiveness to Leukemia Treatment

"Express Mail" Label No. **EV583917128US**
Date of Deposit **February 22, 2005**

I hereby certify that this paper is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Mail Stop AF, Commissioner for Patents, P.O. Box 1450 Alexandria, VA 22313-1450.

By _____
Type Name: **Jane Massey Licata, Reg. No. 32,257**

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

RULE 132 DECLARATION

1. I, Dr. Brian Sorrentino, M.D. am an inventor in U.S. Patent Application Serial No. 09/866,866 filed May 29, 2001 and the parent application U.S. Serial No. 09/584,586, filed May 31, 2000, and am most familiar with the subject matter of this application and the research effort which led to the discovery of the instant invention.

2. In accordance with the methods disclosed in Patent Application Serial No. 09/866,866 and U.S. Patent Application Serial No. 09/584,586, I have generated four independent monoclonal antibodies, designated 5D3, 7A3, 1C5, and 8C2, which recognize an extracellular portion of BCRP in its natural conformation in living cells. As indicated at pages 20-24 and 39 of the '866 application and pages 40-44 of the of the '586 application, these antibodies were generated by cloning a human BCRP cDNA into a Harvey murine sarcoma virus backbone, expressing the cDNA in NIH 3T3 cells (see page 39, lines 1-10 of the '866 application), and immunizing a mouse with the transduced cells (see page 39, lines 12-20 of the '866 application). To test whether the supernatant from the four pre-screened hybridoma clones produce monoclonal antibodies that recognize an extracellular portion of human ABCG2 (*i.e.*, BCRP) on live cells, we first made a human myeloid leukemic cell line over-expressing ABCG2 (AML3/BCRP/6.2) by transducing the AML3 cells with a retrovirus vector (HaABCG2). We then stained the cells with supernatant from the hybridoma clone cultures and a Phycoerythrin (PE)-conjugated secondary rabbit-anti-mouse antibody. The cells were than analyzed in a flow cytometry for PE fluorescence. If an antibody specifically binds ABCG2, the PE fluoresce of AML3/BCRP/6.2 cells stained with the monoclonal antibody would be higher than that of the parental cells that do not express ABCG2 (AML3). As illustrated in Appendix A, each of these antibodies was found to be recognize the extracellular portion of ABCG2; however, whereas 5D3 and 8C2 interacted strongly with ABCG2 in its natural conformation in living cells (see panels to the right of "5D3.F2(2b)" and "8C2.H1(2b)"), the interaction between 1C5 or 7A3 and ABCG2 in its natural conformation in living cells was not as strong (see panels to the right of "1C5.G10(2a)" and "7A3.H7(2a)").

Having definitively generated more than one antibody that recognizes an extracellular portion of BCRP in its natural conformation on living cells, this method is clearly a validated method for generating an antibody to an extracellular portion of

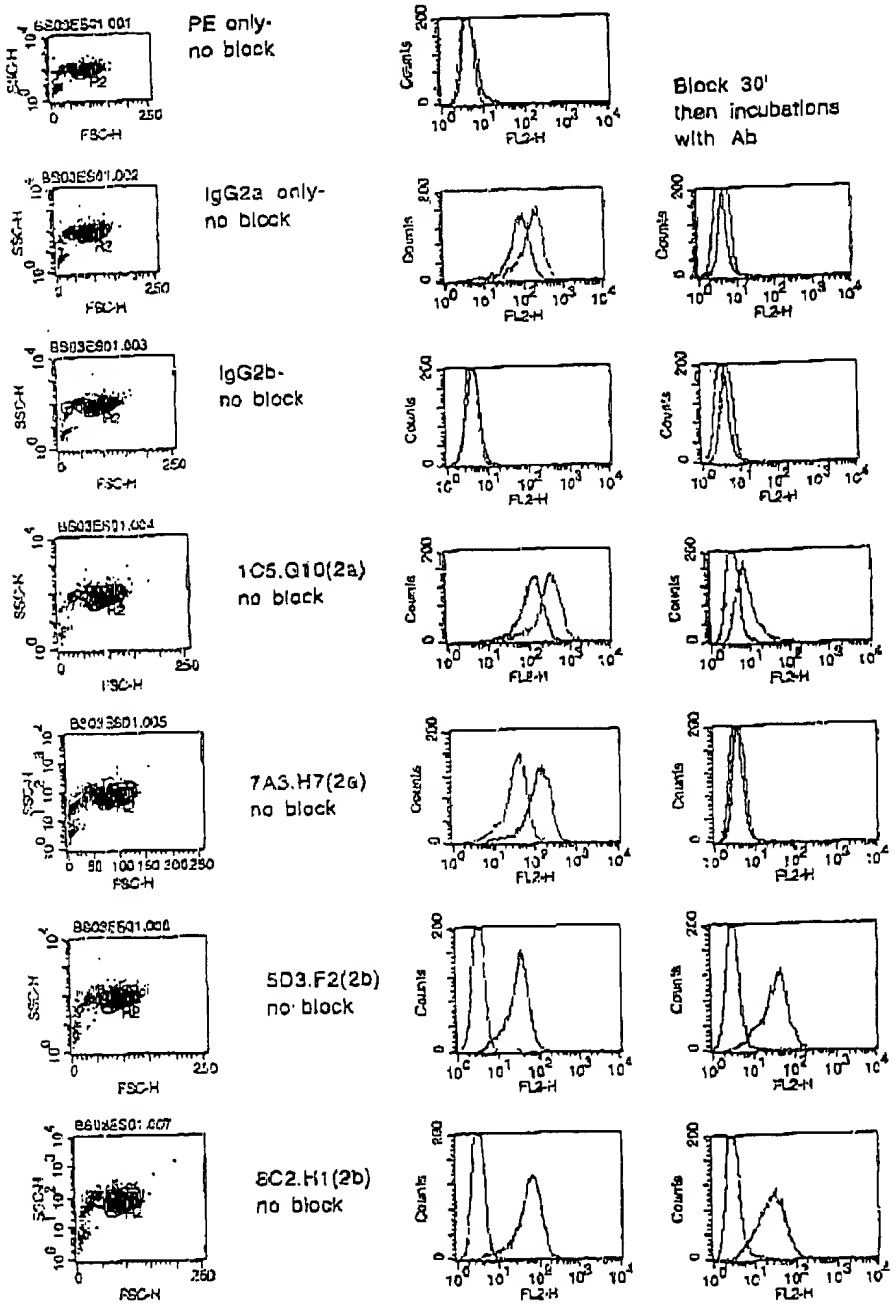
this particular ABC transporter in its natural conformation. I expect that the robustness of this method will allow for routine generation of additional antibodies of the type claimed by simply repeating the procedures disclosed in the '586 and '866 applications.

I hereby declare that all statements herein of our own knowledge are true and that all statements made on information or belief are believed to be true; and further that these statements were made with the knowledge that willful statements and the like so made are punishable by fine or by imprisonment, or both under §1001 of Title 18 or the United States Code, and that such willful statements may jeopardize the validity of the application, any patent issuing there upon, or any patent to which this verified statement is directed.

Date: 2/20/05

Brian Sorrentino, M.D.

EXHIBIT A



Re-Test subcloned sups on AML3 and AML3 BCRP 6.2 with and without blocking agent

Blocking agent = immunoglobulin +serum (100ul)- Rt 30minutesAML3

AML3 NO BLOCK= green
AML3BCRP6.2 NO BLOCK= blue
AML3 +BLOCK= red
AML3BCRP6.2 +BLOCK=

Anne-Marie

Page 1

RESPONSE UNDER 37 CFR 1.116
EXPEDITED PROCEDURE
EXAMINING GROUP 1632

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket No.:	**1340-1-021CIP2 (SJ-0015)**
Inventors:	**Sorrentino and Schuetz**
Serial No.:	**09/866,866**
Filing Date:	**May 29, 2001**
Examiner:	**Li, Qian Janice**
Customer No.:	**31949**
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"Express Mail" Label No. **EV583917128US**
Date of Deposit **February 22, 2005**

I hereby certify that this paper is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Mail Stop AF, Commissioner for Patents, P.O. Box 1450 Alexandria, VA 22313-1450.

By _____
Typed Name: **Jane Massey Licata, Reg. No. 32,257**

Mail Stop AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

SUPPLEMENTAL DECLARATION

1. I, Dr. Balazs Sarkadi, M.D., Ph.D., submit this declaration to supplement my Declaration dated October 27, 2003, wherein I identified myself as an expert in the field of ABC transporters, especially as it pertains to the generation of antibodies to ABC transport proteins including BCRP, as evidenced by my curriculum vitae and list of publications.

2. I have reviewed the Office Action issued in this case dated October 21, 2004. I have further reviewed and understand the prior art methods taught by Ross (U.S. Patent No. 6,313,277) and Mechetner et al. (U.S. Patent No. 5,994,088) as identified by the Examiner in the Office Action.

3. As stated in my previous Declaration, I maintain that at as of the priority date of May 31, 2000 there was no *reliable* method known in the art for producing an isolated antibody that recognizes an extracellular portion of the ABC transporter BCRP in a living cell. The production of antibodies to any ABC transporter can only be evaluated on a case-by-case basis as these proteins, while falling within the superfamily of ABC transporters, have individually distinct topologies, post-translational modifications, and protein-protein interactions which may affect their antigenicity.

4. One of skill in the art of antibody production would have appreciated at the time of filing of present application that, while it would be reasonable to try the various methods known in the art, the reasonable expectation of successfully producing an antibody that recognizes the extracellular portion of BCRP in its natural conformation could not be anticipated.

5. As stated in my previous Declaration, Ross suggests an antibody prepared against a purified BCRP protein. Such a purified protein would not be expected to adopt the three-dimensional structure of BCRP as it is found in the cell membrane where it is a half-transporter that forms a fully functional homodimer. As antibodies can recognize conformational epitopes, the three-dimensional structure is critical for production and recognition of an epitope composed of a specific domain (*e.g.*, extracellular portion) in a particular conformation (*e.g.*, the natural conformation).

6. While the Mechetner et al. reference describes a cell-based method for producing an antibody to an ABC transporter, this single success does not change my view on the need to assess any particular method for producing an antibody to an ABC transporter on a case-by-case basis. Only when an antibody is actually produced to a particular ABC transporter can the method used be validated for the production of an antibody to that particular ABC transporter, in particular when a specific domain (*e.g.*, extracellular portion) and conformation (*e.g.*, the natural conformation) is desired.

7. In my recent publication (Ozvegy-Laczka, et al. (Dec. 2004) *J. Biol. Chem.*, submitted herewith), I have used an antibody produced in accordance with the method of U.S. Patent Application Serial No. 09/866,866, (*i.e.*, 5D3) and shown that the interaction of 5D3 with BCRP in intact cells is dependent upon the actual conformation within the transport cycle of this multidrug resistance protein. In contrast, an antibody generated against an N-terminal intracellular epitope of BCRP (*i.e.*, BXP-21) cannot recognize BCRP in a living cell. Therefore, this method is essential to producing an antibody which recognizes an extracellular portion of BCRP in its natural conformation.

I hereby declare that all statements herein of our own knowledge are true and that all statements made on information or belief are believed to be true; and further that these statements were made with the knowledge that willful statements and the like so made are punishable by fine or by imprisonment, or both under §1001 of Title 18 or the United States Code, and that such willful statements may jeopardize the validity of the application, any patent issuing there upon, or any patent to which this verified statement is directed.

Dr. Balazs Sarkadi, M.D. Ph.D.

Date: 01.17.2005.